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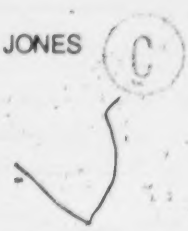
# EFFECTS OF BACTERIOPHAGE AND STORAGE CONDITIONS ON ACTIVITY OF COMMERCIAL STARTER CULTURES

by

TINEKE H. JONES



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SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

FOOD MICROBIOLOGY

DEPARTMENT OF FOOD SCIENCE

EDMONTON, ALBERTA
FALL, 1990

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Permission is hereby granted for you to use the paper "Comparative Evaluation of Bulk Starter Substrates on Activity and Storage of Two Commercial Starter Strains" published in the Journal of Dairy Science 73:1166-1172, as a chapter in your MSc thesis.

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SUBMITTED BY: TINEKE H. JONES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF:

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in

FOOD MICROBIOLOGY

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Date: August 16, 1990

## DEDICATION

This thesis is dedicated to my parents, who instilled in me the values of a good education and gave me the opportunity to pursue it; to my husband Terry, for all his patience, understanding and support he has given me; and to our daughter Melissa, that she may be inspired to reach for her own stars.

ABSTRACT

The potential for use of ultrafiltered (UF) milk retentates and increased milk solids substrates as bulk culture media with respect to starter activtiy, bacteriophage susceptibility and storage stability at refrigeration and freezing temperatures was examined. Internal pH controlled medium (ICM) and 10% reconstituted skim milk (RSM) were used as positive and negative controls, respectively. Eight commercial strains of *Lactococcus lactis* ssp. *cremoris* were evaluated, two of these were used in further study, based on their sensitivity and insensitivity to acidity when grown and stored in 10% RSM. UF retentate R3:1 and 24% RSM provided similar protection against exposure to low pH as ICM after 5 days of storage at 2°C but they lacked phage inhibitory compared with ICM. Loss of activity in 10% RSM after 5 days of storage at 2°C was not plasmid mediated but was due to injury of the cells. One form of injury observed as a result of storage at 2°C was a reduced proteolytic activity that contributed to an increased lag phase. Injury was observed when the pH fell below 4.5 and was caused by an accumulation of lactate ion as opposed to hydrogen ion. Starter cultures were successfully maintained in 10% RSM, 24% RSM and R3:1 for 2 months at -70°C. Neutralization of cultures before freezing reduced loss of proteolytic activity while viability and lactose metabolism were maintained. At present, ICM is a more attractive bulk culture medium than either reconstituted skim milk with increased milk solids or UF milk retentates because of its phage inhibitory properties. M17 is not an ideal medium for phage susceptibility assays because of a gradual loss of phage susceptibility when cultures were repeatedly subcultured in M17 medium.

## ACKNOWLEDGEMENTS

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TABLE OF CONTENTS


CHAPTER PAGE

1. LITERATURE REVIEW

1.1 Introduction ..... 1

1.2 Description and functions of the lactococci in milk fermentations ..... 4

1.3 Metabolism of *L. lactis* and *L. cremoris* ..... 6

1.3.1 Lactose metabolism and regulation ..... 6

1.3.2 Protein metabolism and regulation ..... 9

1.4 Bacteriophage ..... 15

1.5 Acid injury ..... 22

1.6 Bulk starter media ..... 23

1.7 Freezing and storage ..... 27

1.8 Discussion and conclusions ..... 31

References ..... 33

2. COMPARATIVE EVALUATION OF BULK STARTER SUBSTRATES ON ACTIVITY AND STORAGE OF TWO COMMERCIAL STARTER STRAINS

2.1 Introduction ..... 43

2.2 Materials and methods ..... 44

2.2.1 Culture media ..... 44

2.2.2 Composition of milk media ..... 45

2.2.3 Measurement of buffering capacity ..... 45

2.2.4 Preparation of starter organisms in bulk culture media ..... 46

2.2.5 Measurement of starter activity ..... 46

2.2.6 Agglutination behaviour of starter cultures in raw milk and 10% RSM ..... 47

2.3 Results ..... 48

2.4 Discussion ..... 57

References ..... 62

3. STORAGE STABILITY AND BACTERIOPHAGE SUSCEPTIBILITY OF COMMERCIAL STARTER CULTURES IN BULK CULTURE MEDIA

3.1 Introduction ..... 64

3.2 Materials and methods ..... 65

3.2.1 Bacterial strains ..... 65

3.2.2 Storage stability of starter cultures at 2°C ..... 65

3.2.3 Activity of starter cultures ..... 65

3.2.4 Culture media ..... 66

3.2.5 Bacteriophage induction of starter strains ..... 66

3.2.6 Bacteriophage stocks ..... 67

3.2.7 Bacteriophage assays ..... 67

3.2.8 Bacteriophage susceptibility ..... 68

3.2.9 Bacteriophage susceptibility in bulk culture media ..... 68

3.3 Results ..... 69

3.3.1 Storage stability of starter cultures at 2°C ..... 69

3.3.2 Prophage induction ..... 69

3.3.3 Susceptibility of strains to phage 202 and 804 ..... 73

3.3.4 Bacteriophage susceptibility in bulk culture media ..... 74

3.4 Discussion ..... 78

References ..... 81

4. EFFECT OF STORAGE CONDITIONS ON ACTIVITY AND VIABILITY OF STARTER CULTURES

4.1 Introduction ..... 82

4.2 Materials and methods ..... 83

4.2.1 Bacterial strains ..... 83

4.2.2 Preparation of cultures for freezing ..... 83

4.2.3 Starter activity ..... 84

4.2.4 Turbidity measurement of starter cultures ..... 84

4.2.5 Measurement of proteolytic activity ..... 84

4.2.6 Treatment of cultures for determination of acid sensitivity ..... 85

4.3 Results ..... 86

4.4 Discussion ..... 99

References ..... 105

5. GENERAL DISCUSSION AND CONCLUSIONS ..... 107

References ..... 111

LIST OF TABLES


PAGE

Table 2.1 Proximate composition of reconstituted skim milk and UF bulk culture media ..... 49

Table 2.2 Rank order of mean time for *L. cremoris* strains 108 and 205 to achieve pH 4.6 in 10% RSM after storage in different media and significance of difference among means determined at the 95% confidence level using Scheffe's test ..... 56

Table 2.3 Final pH and amount of acidity produced during growth of *L cremoris* strain 108 and 205 in bulk culture media at 32°C for 16h ..... 58

Table 2.4 $Log_{10}$ of colony forming units (CFU)/mL for *L. cremoris* strain 205 grown at 32°C for 16 h and stored at 2°C in different bulk culture media ..... 59

Table 3.1 Activity (Δ%TA) of six strains of *L. cremoris* during storage at 2°C for 5 days in different bulk culture media ..... 70

Table 3.2 Viability ($log_{10}$ of CFU/mL) of 6 strains of *L. cremoris* during storage at 2°C for 5 days in different bulk culture media ..... 71

Table 3.3 Phage titre ($log_{10}$ PFU/mL) of phage 202 when grown on *L. cremoris* strains 205 and 777 in different bulk culture media at 30°C for 16 h ..... 75

Table 3.4 Activity (Δ%TA) of *L. cremoris* strains 205 and 777 after inoculation in different bulk culture media in the presence and absence of phage 202 ..... 76

Table 3.5 Activity (Δ%TA) of *L. cremoris* strains 205 and 777 after growth in different bulk culture media in the presence and absence of phage 202 ..... 77

Table 4.1 Activity (Δ%TA) of *L. cremoris* strain 108 before and after storage at -70°C in different substrates with and without neutralization ..... 87

Table 4.2 Activity (Δ%TA) of *L. cremoris* strain 205 before and after storage at -70°C in different substrates with and without neutralization ..... 88

Table 4.3 Viability ($log_{10}$ of CFU/mL) of *L. cremoris* strain 108 before and after storage at -70°C in different substrates with and without neutralization ..... 89

Table 4.4 Viability ($log_{10}$ of CFU/mL) of *L. cremoris* strain 205 before and after storage at -70°C in different substrates with and without neutralization ..... 90

Table 4.5 Effect of lactic acid and HCl on the activity and viability of *L. cremoris* strain 205 stored at 2°C for 5 days..... 98

Table 4.6 Activity (Δ%TA) and viable count of *L. cremoris* strain 205 grown in 10% RSM for 16 h and stored at different pH levels at 2°C for 5 days..... 100

Table 4.7 Activity (Δ%TA) in 10% RSM by *L. cremoris* strain 205 after growth in 10% RSM and storage at different pH levels at 2°C for 5 days..... 101

## LIST OF FIGURES


PAGE

Fig 2.1 Buffering capacity (dB/dpH) of reconstituted milk media (10%, 20% and 24%) determined as a function of change in pH by titration with 1 N HCl ..... 50

Fig 2.2 Buffering capacity (dB/dpH) of ICM compared with 10% RSM determined as a function of change in pH by titration with 1 N HCl ..... 51

Fig 2.3 Effect of growth and storage in 10% RSM at 2°C for 5 days on activity of *L. cremoris* strain 108 ..... 53

Fig 2.4 Effect of growth and storage in 10% RSM at 2°C for 5 days on activity of *L. cremoris* strain 205 ..... 54

Fig 2.5 Effect of growth and storage in different bulk media at 2°C for 5 days on activity of *L. cremoris* strain 205 ..... 55

Fig 3.1 Growth of *L. cremoris* strain 108 after exposure to UV light ..... 72

Fig 4.1 Growth of *L. cremoris* strain 108 at 30°C before and after 56 days of storage at -70°C with and without neutralization ..... 91

Fig 4.2 Growth of *L. cremoris* strain 205 at 30°C after 56 days of storage at -70°C in different substrates ..... 92

Fig 4.3 Effect of time of storage at -70°C in 10% RSM on proteolytic activity of *L. cremoris* strain 108 in 10% RSM at 30°C ..... 94

Fig 4.4 Effect of time of storage at -70°C in 10% RSM on proteolytic activity of *L. cremoris* strain 205 in 10% RSM at 30°C ..... 95

Fig 4.5 Effect of storage medium on proteolytic activity of *L. cremoris* strain 108 in 10% RSM at 30°C after storage at -70°C for 56 days ..... 96

Fig 4.6 Effect of storage medium on proteolytic activity of *L. cremoris* strain 205 in 10% RSM at 30°C after storage at -70°C for 56 days ..... 97

Fig 4.7 Proteinase activity of *L. cremoris* strain 108 and 205 after storage in 10% RSM at 2°C for 5 days ..... 102

# 1. LITERATURE REVIEW

## 1.1 Introduction

Cheese has been made as a food for thousands of years. The origin of cheesemaking is not known but it was most likely made accidentally. Cheese making began and developed as an art and remained so until the advent of the knowledge of microbiology and chemistry during the past century (Pederson, 1979). Traditionally, milk was left to sour naturally before it was used as a starter for cheese making. In 1841, C. J. Fuchs postulated the theory that the formation of acid in milk was a biological process. Louis Pasteur, in 1858, was the first to prove Fuchs' theory by showing that acid production in milk was caused by microorganisms, which he called *"levure lactique"* (Lundstedt, 1983). In 1873, Joseph Lister named the lactic acid bacteria named *"Bacterium lactis"*, commonly known as *Streptococcus lactis* for many years and now known as *Lactococcus lactis* subsp. *lactis* (referred to as *L. lactis* in this thesis).

The use of traditional starter cultures, originating from naturally soured milk or cream, was not without problems. The starter cultures were prone to failure, could promote undesirable side effects and could cause considerable variation to the quality of the end product. All of these problems could be attributed to the lack of knowledge in microbiology. Since the early 1900's, starter cultures have been subjected to intensive research and their behaviour and metabolism are now well understood.

The selection of starter cultures and the need for predictable activity has become of great importance to the dairy industry, because of the large volumes of milk that are made into cheese and the need for standardizing the quality of final product. In this multi-million dollar industry, the classification, maintenance, preservation, propagation and optimization of starter cultures is of tremendous economic importance.

Over the last 50 years research has been done on all aspects of dairy starter cultures, ranging from starter culture preservation to genetic manipulation of the bacteria. However, the common thread throughout these studies is starter activity. Starter activity must be optimized and predictable. There are many factors that influence starter activity, including the stability of the starter strain, presence of inhibitors in milk, growth temperature, bulk culture media, bacteriophages and storage practices.

One important factor is the stability of the properties and fermentation characteristics of the starter organisms themselves. Strains of starter bacteria possess characteristics which are hereditary and are normally retained when the culture is propagated. In the case of single strain starters, continuous transfer may lead to a culture containing a proportion of slow acid or slow proteolytic variants (Citti *et al.*, 1965). When the "slow" variants reach significant proportions in the culture, the overall starter activity becomes impaired. Extrinsic inhibitors may be present in milk such as nisin produced by certain strains of *L. lactis* in raw milk, antibiotics from mastitis treatment and residues of sanitizers used in the cleaning of milk and dairy equipment. Growth temperature also influences starter activity. The optimum growth temperature for lactococci is generally regarded to be 30°C (Sandine, 1985).

Traditionally, reconstituted skim milk (RSM) was used to prepare bulk starter cultures. However, starters tend to lose activity on prolonged incubation or storage in 10% RSM, due to the exposure of the culture to pH levels below 5.0. Harvey (1965) found that incubation of *L. lactis* in a broth medium below pH 5.0 resulted in a marked reduction in the specific activity of cytoplasmic enzymes. The growth of starter cultures in milk is limited by the lactic acid they produce and cell concentrations up to ten-fold higher can be achieved if some form of neutralization is used (Davies and Gasson, 1984).

One of the most important factors affecting starter activity is the presence of bacteriophage. These bacterial viruses attack and destroy starter cultures, resulting

in decreased or failure of lactic acid production. The presence of bacteriophage in dairy starter cultures was first reported by Whitehead and Cox (1936). In the 1970's, bulk culture systems were developed that combined neutralization of lactic acid with bacteriophage control. Richardson *et al.* (1977) developed a low cost phage inhibitory medium which was composed of dilute liquid whey to which yeast extract and phosphates were added. A pH of 6.0 is maintained by the controlled addition of ammonium hydroxide which is monitored using external pH control. The phosphates complex the available calcium and thus inhibit bacteriophage. Sandine and Ayers (1983) developed Phase 4, which is an "internal" pH controlled phage inhibitory medium. This medium is composed of sweet whey, yeast extract, and phosphate and citrate buffers. The buffering system ensures that the pH does not drop below pH 5.0, thus preventing acid injury of the bacteria. This medium also contains chelating agents that tie up available calcium, thus inhibiting the multiplication of calcium-requiring phage (Mermelstein, 1982). Because the pH is maintained above 5.0 in both the external and internal pH controlled media, starter organisms grow to higher numbers and maintain their activity over a longer period of time compared with organisms grown in RSM.

More recently, ultrafiltered (UF) milk retentates have been investigated as a potential bulk starter medium (Mistry and Kosikowski, 1986). UF retentates have a natural built-in buffering system due to the increased mineral and milk protein content. The high buffering capacity of UF milk is regarded as a drawback for the manufacture of cheese, because starter cultures must be very active and produce large amounts of lactic acid to overcome the natural buffering capacity and to reach the desired pH for cheese making (Hickey *et al.*, 1983; Mistry and Kosikowski, 1985b). However, the natural buffering capacity of UF skim milk could be exploited for the production and maintenance of active bulk starter cultures during storage. UF

retentates have the potential to provide a protective environment for starter organisms by minimizing acid injury, which allows the starter to grow to greater numbers.

Methods used for storage of starter cultures can greatly influence their activity. Mature starter cultures suffer damage if they are exposed to the acidity they have produced and therefore, for long term storage and preservation, frozen, frozen concentrated or freeze dried cultures are widely used. Much research has been done on the production of such cultures. It appears that the optimum temperature for long term storage is -196°C, i.e. liquid nitrogen, whereas storage at -20° C or above are the most damaging to the cells (Gilliland, 1985).

The objectives of this research were to examine the potential for use of UF retentates and increased milk solids substrates as bulk culture media with respect to starter activity, bacteriophage susceptibility and storage stability at refrigeration and freezing temperatures.

## 1.2 Description and functions of the lactococci in milk fermentations

Lactic acid bacteria used in milk fermentations may be divided into two general categories based on their optimum growth temperatures: mesophiles with growth optima of 30°C and thermophiles with growth optima >37°C. The mesophilic starter cultures include two genera: *Lactococcus* and *Leuconostoc*. The lactococci are gram positive cocci with a diameter ranging from 0.5 to 1.0 µm, usually found in pairs or short chains and they react with Lancefield's group N antisera. The group antigenic determinant is a glycerol teichoic acid containing galactose phosphate, which is an intracellular constituent. They can be distinguished from streptococci and enterococci by their ability to grow at 10°C but not at 45°C. They grow in 4% NaCl but not in 6.5% NaCl. Their nutritional requirements are complex, requiring 4 or 5 of the B-

vitamins and 10 to 13 amino acids. *Lactococcus lactis* is a homofermentative bacterium, producing mainly L-lactic acid from glucose or lactose. The final pH in glucose broth ranges from 4.0 to 4.5. Litmus milk is reduced and an acid clot is formed (Deibel and Seeley, 1986).

The three subspecies of *Lactococcus lactis* used for starter cultures are *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *diacetylactis* (Schleifer, 1987), subsequently referred to as *L. lactis*, *L. cremoris* and *L. diacetylactis*, respectively. *L. diacetylactis* is differentiated from *L. lactis* and *L. cremoris* by its ability to metabolize citrate, which is a plasmid mediated trait (Kempler and McKay, 1979). *L. cremoris* is distinguished from *L. lactis* by its inability to produce ammonia from arginine because it does not produce the enzyme arginine deaminase and, in some strains, loss of ability to produce a second enzyme, ornithine transcarbamylase (Crow and Thomas, 1982). Growth at 40°C can be used as another differentiating characteristic. *L. lactis* grows at 40°C while *L. cremoris* does not. Nisin and diplococcin are antibiotic-like substances produced by some strains of *L. lactis* and *L. cremoris*, respectively. Certain strains of *L. diacetylactis* produce an unnamed antibiotic (Sandine, 1985).

Starter cultures influence the flavour, body and texture of fermented dairy products. The major functions of starter cultures in milk fermentations are acid production, flavour and aroma production, proteolysis and preservation. The primary role of starter cultures is to convert lactose to lactic acid. Lactic acid causes the formation of an acid casein curd at pH 4.6 and aids the expulsion of whey from the curd (syneresis). Acid production also aids rennet action and subsequent coagulum formation. The conversion of lactose to lactic acid also makes the product more acceptable for lactose-intolerant individuals. *L. lactis* and *L. cremoris* are mainly used for acid production while *L. diacetylactis* is used for both acid production and flavour development. *L. diacetylactis* is capable of fermenting citrate, producing $CO_2$ and the

flavour compounds diacetyl and acetaldehyde. Starter cultures are a source of proteolytic enzymes, thus contributing to the cheese ripening process. Proteolytic enzymes break down milk proteins and give cheese its characteristic texture. Starter cultures also play a role in the preservation of dairy products because the lactate produced prevents or discourages growth of undesirable spoilage and pathogenic microorganisms.

## 1.3 Metabolism of *L. lactis* and *L. cremoris*

### 1.3.1 Lactose metabolism and regulation

Lactococci are nutritionally fastidious and have very limited biosynthetic and catabolic activities. Carbohydrate metabolism is homolactic and cells do not possess a cytochrome system for electron transport, or enzymes to operate anaplerotic pathways or the tricarboxylic cycle (Lawrence *et al.*, 1976). Lactose fermentation is a three stage process involving: (a) the vectorial translocation and phosphorylation of lactose by a lactose-specific phosphotransferase system (PTS); (b) catabolism of lactose-6-phosphate (lactose-6P) by energy yielding pathways, and (c) efflux of lactic acid from the cell (Thompson, 1987).

Lactose is taken up by a lactose-specific phosphoenol pyruvate (PEP)-dependent PTS (McKay *et al.*, 1970). The lactose-6P inside the cell is hydrolyzed by phospho-ß-galactosidase (P-ß-gal) into galactose-6-phosphate (gal-6P) and glucose (Johnson and McDonald, 1974). Glucose is phosphorylated and fermented via the glycolytic pathway which is characterized by the splitting of fructose-1,6-diphosphate (FDP) with aldolase into two triose phosphate moieties which are further converted to lactic acid.

Gal-6P is metabolized by the D-tagatose-6-phosphate pathway which involves the conversion of gal-6P to D-tagatose-6-phosphate (tag-6P) by D-galactose-6-

phosphate isomerase. It is further phosphorylated by D-tagatose-6-phosphate kinase to D-tagatose-1,6-diphosphate (TDP) and split into two triose phosphate molecules by D-tagatose-1,6-diphosphate aldolase, which then enter the glycolytic cycle. The three enzymes involved in this pathway are inducible (Bissett and Anderson, 1974) and have been linked to a 33 Mdal plasmid in *L. lactis* H1 (Crow *et al.*, 1983).

Strains lacking a PEP:PTS can still metabolize lactose, but at much slower rates, by utilizing a lactose permease and ß-galactosidase (Thomas, 1976). Galactose is metabolized via the Leloir pathway in which it is phosphorylated to gal-1-P by galactokinase and is converted to glucose-6-phosphate through a glucose-1-phosphate intermediate catalyzed by glucose-1-phosphate transferase and an epimerase. The Leloir pathway is repressed in lactococci that are grown on lactose when the PEP:PTS and D-tagatose-6-phosphate pathway are operating (Bissett and Anderson, 1974). Homolactic fermentation of lactose by lactococci yields 4 moles of ATP per mole of lactose.

Lactose utilization via a PEP-dependent PTS system was first demonstrated in lactococci by McKay *et al.* (1970). The PTS system is composed of Enzyme I (EI), HPr, Factor III-lac (FIII-lac) and Enzyme II-lac (EII-lac). EI and HPr are soluble cytoplasmic components that are constitutively synthesized. FIII-lac is a soluble cytoplasmic component and EII-lac is a membrane bound component, both are galactoside-specific (McKay, 1985). FIII-lac, EII-lac and P-ß-gal are induced in the presence of galactose or gal-6P (Morse *et al.*, 1968). They have been linked to a 36 Mdal plasmid in *L. cremoris* B1 (Anderson and McKay, 1977).

Regulation of lactose metabolism in lactococci involves both "coarse" control of enzyme synthesis exerted at the level of gene expression in the presence of appropriate inducers, and "fine" control of enzyme activity in response to fluctuations in concentrations of intracellular effector molecules. "Fine" control of lactose metabolism may involve regulation by end products, precursor activation, allosteric

enzymes, the energy status of cells and the balance of oxidized and reduced forms of pyridine nucleotides (Lawrence *et al.*, 1976).

PEP is found at a branch point from which it is committed to either lactose transport or ATP synthesis. PEP provides a tight linkage between lactose transport and its subsequent metabolism, because it serves as a high energy phosphate donor for lactose transport and as a reactant in sugar fermentation. The conversion of PEP to lactic acid involves the allosteric enzymes pyruvate kinase (PK) and lactate dehydrogenase (LDH) both of which are activated by FDP (Jonas *et al.*, 1972; Collins and Thomas, 1974). PK is the key regulator of glycolysis in lactococci (Thomas, 1976). The control of PK activity may regulate the PEP concentration and hence provides a coupling between lactose transport via the PEP-dependent PTS and the subsequent metabolism of lactose. During glycolysis, when both PTS and PK are most active, cells of *L. lactis* have a low PEP potential and contain high levels of FDP and low levels of inorganic phosphate ($P_i$). During starvation the cells are depleted of FDP but maintain high levels of $P_i$ and PEP intermediates resulting in an inoperative PTS and PK (Thompson, 1987). Fluctuations in the ratio of FDP:$P_i$ modulate the activity of PK which in turn dictates the rate of lactose fermentation by the PTS:glycolysis cycle (Fordyce *et al.*, 1984). Regulation of LDH activity has the potential to control the overall activity of the pathways for carbohydrate metabolism during anaerobic growth. Its activity is dependent on pH and the presence of FDP and TDP (Jonas *et al.*, 1972). Regulation of LDH activity may ensure a low intracellular pyruvate concentration so that, under anaerobic conditions, other pathways of pyruvate metabolism are not expressed and fermentation is homolactic (Thomas, 1976).

The glucose produced by P-ß-gal as a result of lactose hydrolysis was presumed to be phosphorylated by glucokinase (GK) because high levels of constitutive GK were found in *L. lactis* (Thompson and Chassy, 1985) Significant concentrations of free glucose were not detected in the medium during normal growth on lactose (Thompson,

1987). However, since the discovery of the PEP:PTS system in *Staphylococcus aureus* (Kundig *et al.*, 1964), the possible role of PTS in phosphorylation of intracellular glucose generated from lactose has been debated. Thompson (1979) suggested that PEP served as the phosphate donor by either intracellular phosphorylation by the mannose-PTS or following exit and re-entry of glucose by the mannose-PTS. Thompson and Chassy (1985) showed that intracellular phosphorylation of glucose occurs by the mannose-PTS in *L. lactis*.

Lactic acid production is known to continue after bacterial growth has ceased. In lactococci this uncoupling of lactic acid and ATP production from cell growth occurs during starvation in the presence of lactose (Thomas and Batt, 1968), nitrogen limitation of proteinase deficient variants in milk (Pearce *et al.*, 1974) and during incubation at pH values below 5.0 (Turner and Thomas, 1975).

Enzymes required for the conversion of glucose-6-phosphate to ribose-5-phosphate via the pentose phosphate pathway have been discovered at low levels compared with glycolytic enzymes in lactococci (Oram and Reiter, 1966; Vakil and Shahani, 1970; Demko *et al.*, 1972). The main functions of this pathway are to supply pentose phosphate for nucleotide synthesis and reducing equivalents ($NADPH_2$) for biosynthesis, including fatty acids (Lawrence *et al.*, 1976).

### 1.3.2 Protein metabolism and regulation

Starter bacteria possess a proteolytic system consisting of proteinases and peptidases which act together to hydrolyze milk protein, making the amino acids (AAs) and peptides available for growth, as well as creating the characteristic rheological and organoleptic properties of foods (Law and Kolstad, 1983). The lactococci have a complex proteolytic system in terms of numbers and types of different proteinases and peptidases. However, they are weakly proteolytic compared with other groups of bacteria such as *Bacillus*, *Proteus*, *Pseudomonas* spp, and the coliform bacteria (Law

and Kolstad, 1983). The proteolytic system of lactococci has been the subject of intensive research since 1970, however, generalizations and comparisons are difficult to make because of the wide range of different strains studied, the different growth conditions used and the variety of methods employed for release or extraction of the proteinases (Thomas and Pritchard, 1987). The proteolytic system of *L. cremoris* consists of cell wall proteinase(s), membrane-bound peptidases and an intracellular system which includes a proteinase and peptidases (Exterkate, 1975).

Strains of *L. cremoris* may contain up to three types of cell wall-associated proteinases ($P_I$, $P_{II}$ and $P_{III}$), classified according to their temperature and pH optima (Exterkate, 1976). There are marked strain variations with respect to the numbers and combinations of these enzymes. Proteinases isolated from cell walls have pH optima around 5.5 to 6.5 and they are activated or stabilized by calcium ions (Exterkate, 1979a). Exterkate and De Veer (1987) characterized cell wall-associated proteinase $P_I$ isolated from *L. cremoris* HP. The enzyme is a serine proteinase with a pH optimum around 6.4. A hypothesis was postulated that $P_I$ exists as a complex consisting of two distinct proteolytically active subunits and one or more proteins. These are held together tightly by interactions mediated by calcium bridges and possibly by hydrophobic interactions which stabilize the enzyme in its most efficient conformation. Proteinases responsible for the degradation of extracellular proteins are associated with the cell wall in most strains, with the exception of *L. cremoris* ML1, which secretes an extracellular proteinase into its growth medium (Exterkate, 1976).

Cell wall-associated proteinases are responsible for breaking down the extracellular protein, while intracellular proteinases are important in the turnover of denatured or defective proteins, the activation of zymogens (preenzymes) and the termination of newly synthesized proteins (Law and Kolstad, 1983). Upon cell lysis they may contribute to ripening and flavour development by breaking down milk

proteins and peptides (Thomas and Pritchard, 1987). *L. lactis* and *L. cremoris* are believed to possess intracellular proteinases, however, their relative distribution and exact locations are not known (Pearce *et al.*, 1974; Exterkate, 1975, 1976). Two distinct types of intracellular proteinase activity, one with an optimal temperature around 30 to 40°C, the other at 5 to 10°C, have been reported in *L. lactis* (Akuzawa *et al.*, 1985).

Cultures grown in milk have much higher levels of cell wall-bound proteolytic activity than cultures grown in broth media which contain high levels of free amino acids (AAs) and peptides (Exterkate, 1976; 1979a). This is attributed to the requirement for calcium ions for accumulation of proteinases in the cell wall and to the repressive effect of free AAs or peptides present in the broth medium. Prolonged accumulation of proteinase $P_{III}$ in the cell wall of *L. cremoris* AM1 occurs in the absence of messenger ribonucleic acid (mRNA) synthesis. This process involves *de novo* protein synthesis, indicating that accumulation is supported by preformed proteinase-specific mRNA, which is either intrinsically long lived or it is stabilized following its transcription (Exterkate, 1979a). Translation of newly synthesized proteinase-specific mRNA is regulated by the extracellular concentration of AAs and/or peptides. Low molecular weight peptides exert a more potent repressive effect than free AAs on proteinase production in *L. cremoris* AM1 (Exterkate, 1985).

Exterkate (1985) proposed a dual control model for the regulation of cell wall proteinase synthesis by *L. cremoris* AM1 during growth of the organism in milk. One form of control inhibits proteinase-specific mRNA synthesis when the endogenous AA pool exceeds the requirement for protein synthesis. The second level of control involves the synthesis of a translational inhibitor which is produced if the AA supply is greater than required for protein synthesis. Because protein is the only nitrogen source in milk, continuous synthesis of cell wall-bound proteinases and membrane-

bound peptidases must be insured to provide the cell with sufficient peptides and AAs for balanced growth (Exterkate, 1979a).

Plasmid-mediated production of proteinase enzymes was first suggested in the early 1970's, because of the high rate of spontaneous loss of proteolytic activity observed, as well as presumptive data from plasmid curing experiments (Molskness *et al.*, 1974; Pearce *et al.*, 1974). McKay and Baldwin (1975) were the first to provide physical evidence that proteinase activity is associated with plasmids. A $Lac^+Prt^-$ variant of *L. lactis* C2 had lost a 12.5 Mdal plasmid (Klaenhammer *et al.*, 1978). In *L. lactis* strains ML3, C10 and M18, loss of 33, 40 and 45 Mdal plasmids, respectively, was accompanied by loss of both lactose and proteinase activities (Kuhl *et al.*, 1979). *L. cremoris* strains are less readily cured of proteinase activity (Larsen and McKay, 1978), although a $Prt^-$ variant of *L. cremoris* HP lost an 8.5 Mdal plasmid and a $Lac^+Prt^-$ variant of *L. cremoris* ML1 lost a 2.2 Mdal plasmid.

The spontaneous loss of lactose and proteinase activities has led to speculation that these traits are linked and may reside on the same plasmid (Kuhl *et al.*, 1979). The proteinase gene has been mapped on a 33 Mdal plasmid in *L. lactis* 712 (Gasson, 1983), and similarly on a 17.5 Mdal plasmid in *L. crembris* Wg2 (Kok *et al.*, 1985). It is unclear whether cell wall-bound extracellular and unbound intracellular proteinases are encoded on separate DNA species. A proteinase deficient variant of *L. cremoris* HP lost two cell wall proteinases and intracellular proteinase (Exterkate, 1976), while proteinase deficient variants of *L. lactis* lost cell wall proteinases but retained their intracellular proteinase activity, which suggests separate gene locations (Pearce *et al.*, 1974). It is feasible to have intracellular proteinases encoded on stable chromosomal DNA because their loss would be more damaging to cellular metabolism than the loss of the plasmid encoded surface-bound proteinases (Law and Kolstad, 1983).

A wide range of different types of peptidases have been described in lactococci including amino peptidases, dipeptidases, tripeptidases and aryl amidases (Thomas and Mills, 1981; Law and Kolstad, 1983; Marshall and Law, 1984; Van Boven and Konings, 1986). These enzymes have exopeptidase activity (act on the ends of peptides) and they are found intracellularly, in the cell membrane or associated with the cell wall. Peptidase activity of lactococci is not affected by the composition of the growth medium (Mou *et al.*, 1975; Schmidt *et al.*, 1977). Mou *et al.* (1975) suggested that the specificity of peptidases found in lactococci is wide enough to ensure the hydrolysis of all casein-derived peptides to free AAs. The peptidases display a large degree of diversity in their pH and temperature optima and in their molecular weights, although they all appear to be EDTA-sensitive metallo-enzymes (Law and Kolstad, 1983). Optimum catalytic activity is achieved when the peptidases interact with the cell membrane (Exterkate, 1979b).

Peptide hydrolysis and peptide transport are thought to be association with the cell membrane in *L. cremoris*, because peptidases have been located near the outside surface of the cell membrane and in the cell membrane itself (Exterkate, 1984). Knowledge of the peptide transport systems in lactococci is very limited but it is clear that both extracellular and intracellular peptidases contribute to protein catabolism and AA nutrition. It is not known whether the bulk of AAs used for starter growth in milk enters the cell in free form or as small peptides (Thomas and Pritchard, 1987). Peptide uptake can be favoured over AA uptake if (a) peptide hydrolysis is linked to peptide transport; (b) peptides are transported by a peptide carrier and hydrolyzed internally; (c) after uptake and hydrolysis of peptides some AAs efflux, creating a proton motive force (Van Boven and Konings, 1986). The upper size limit for peptide transport through lactococcal membranes is five or six AA residues (Law, 1978; Rice *et al.*, 1978), implying that larger peptides require hydrolysis before uptake.

The potential sources of nitrogen for starter growth in milk are casein, whey proteins and other low molecular weight nitrogen compounds. The caseins have an open, largely random structure which makes them readily susceptible to proteolysis. The whey proteins are globular molecules with a high degree of secondary and tertiary structure, making them more resistant to proteolysis (Thomas and Pritchard, 1987). The free AAs present in fresh milk are well below the minimum requirements for growth of the lactococci to high cell densities, but they are an important source of nitrogen for growth to densities of 8 to 16% of the maximum in coagulated milk. After that the AAs must be supplied from other sources (Thomas and Mills, 1981). Peptides of molecular weight <1500 provide a further source of AAs (Law and Kolstad, 1983) but, as cell density increases, cells become more dependent on their proteolytic activities to supply peptides from milk proteins (Thomas and Mills, 1981).

Because of the variation in numbers of different enzymes, their specificity and cellular location, a general pathway for milk protein utilization will be discussed. Possession of cell wall-bound neutral and acid proteinases (Exterkate, 1975) allows cells to hydrolyze protein molecules into oligopeptides, which after further degradation by cell wall peptidases, may be small enough to diffuse through the cell wall. A peptide transport system is required in *L. cremoris*. Some strains cannot utilize dipeptides because of the absence of dipeptide transport systems. *L. lactis* strains are less dependent on a peptide transport system because they possess cell wall-bound peptidases (Law, 1977). The peptides are further degraded by membrane-bound peptidases before being actively transported through the cytoplasmic membrane, because the size limit for uptake is five to six residues (Law, 1978; Rice *et al.*, 1978). Once inside the cell, the peptides are hydrolyzed to free AAs by a wide range of intracellular peptidases (Mou *et al.*, 1975).

Proteolytic enzymes produced by lactococci are also important in the ripening of cheese. Their activity can produce defects as well as desirable changes. Proteolysis

is essential for the conversion of the springy curd of unripe cheeses to inelastic textures of mature cheeses, which is attributed to the breakdown of alpha-casein (Law and Kolstad, 1983). The later stages of proteolysis produce small peptides and AAs which are involved in flavour development of cheese. The most important function of proteinases is the slow degradation of ß-casein and large rennet-derived polypeptides to small peptides and AAs (Law and Kolstad, 1983; Thomas and Pritchard, 1987). Casein molecules, especially ß-casein, have a high content of hydrophobic residues (Visser et al., 1983a, b) giving rise to the formation of bitter peptides. These bitter peptides have molecular weights ranging from 1,000 to 12,000 (Law and Kolstad, 1983). Exterkate (1976) suggested that enzyme specificity is involved. The level of bitter peptides depends on their rate of formation relative to their rate of degradation, which is influenced by the peptidase activity in the cells and by the rate of release of peptidases from lysing cells. The rate of proteolysis by lactococci in cheese is influenced by the stability of proteolytic enzymes (Cliffe and Law, 1979), salt concentration (Stadhouders *et al.*, 1983) and ripening temperature (Stadhouders and Hup, 1975). Richardson *et al.* (1983) investigated the possibility of using Prt$^-$ variants of *L. cremoris* as cheese starters because it is believed that an increase in cheese yield can be obtained through the use of high concentrations of less proteolytic strains. Advantages of Prt$^-$ strains are the following: (a) acid production is uncoupled from cell growth, (b) acid production is consistent in the cheese vat, (c) less casein is hydrolyzed, (d) bacteriophage problems are eradicated, and (e) chances for bitter flavour production are reduced (Thunell and Sandine, 1985).

## 1.4 Bacteriophage

Bacteriophages (phages) are viruses that attack and destroy bacteria. They are of major concern to the dairy industry because their action can be responsible for devastating economic losses. Phage infection of starter cultures can lead to complete

loss of vats of cheesemilk or slow acid production resulting in cheese with a high pH, lactose content and redox potential and low lactic acid content (Mullen, 1986). Whitehead and Cox (1936) were the first to report the presence of phage in starter cultures and since that time research efforts have concentrated on the sources of phage, methods for their control and genetic manipulation of starter cultures to make them phage resistant.

Starter bacteria are susceptible to attack by specific phage types. At least 20 phages with different ultrastructure have been identified from the lysates of over 100 lactococcal strains (Teuber and Lembke, 1983). Both isometric and prolate polyhedral head shaped phages have been observed (McKay and Baldwin, 1973; Huggins and Sandine, 1977; Heap and Jarvis, 1980; Terzaghi and Sandine, 1981). Isometric phages are isolated more frequently and they have a narrow host range. Prolate phages have a wider host range (Heap and Jarvis, 1980). Latent (or incubation periods) of phages in reconstituted skim milk (RSM) range from 23 to 56 min at 30°C to 16 to 44 min at 37°C and burst sizes range from 2 to 113 particles at 30°C to 1 to 139 particles at 37°C (Keogh, 1973; Pearce *et al.*, 1970).

Very little research has been done on regulation and control of lactococcal phages, however, phage lambda (specific for *Escherichia coli*) has been studied in detail and its control and regulation are well understood. Phages alternate between two different life cycles: lytic and lysogenic. In the lytic cycle, after the phage DNA is injected into the host cell, the phage genes are transcribed in a set order. The DNA is replicated many times and coat protein is produced. The host cell is lysed to release the progeny phage (Lewin, 1987). Phage is not produced in the lysogenic cycle. Prophage is phage DNA that has been integrated into the bacterial chromosome and it is replicated with the nuclear material of the bacterial cell. The surviving bacteria are known as lysogens. A lysogen usually carries only one copy of a particular prophage on its chromosome, because prophage gives the cell immunity to infection by phage of the

same type (Lewin, 1987). Prophage can be activated and excised from the bacterial chromosome spontaneously or by induction with UV light or mitomycin C. When activation occurs, the host cell enters a lytic cycle and progeny phage are released (Snyder *et al.*, 1985). Progeny phage may either repeat the lytic cycle or enter the lysogenic state. This depends on the relative activities of the genes coding for viral replication and those coding for lysogenization, which are influenced by environmental conditions (Mathews, 1971).

In a lysogenic bacterium, phage genes are kept inactive by the product of a repressor gene. This gene codes for a repressor protein that is synthesized on a continuous basis, initially by the infecting phage and subsequently by the prophage. The repressor gene is usually the only prophage gene which is expressed (Snyder *et al.*, 1985). Maintenance of the lysogenic state requires continuous synthesis of the repressor protein in quantities sufficient to inhibit phage replication. Induction of lysogenic prophage to enter the lytic cycle is caused by cleavage of the repressor protein (Lewin, 1987). When the level of active repressor protein drops below the level required to maintain lysogeny, the prophage is excised from the host chromosome and enters the lytic cycle (Mathews, 1971). The lysogenic and lytic cycles are closely related, both of them involve the expression of the *N* and *cro* genes (Lewin, 1987). The *cro* gene codes for an antirepressor protein which prevents the synthesis of the repressor protein, thus preventing the establishment of lysogeny. The "decision" to enter the lysogenic or lytic cycle depends on whether the repressor or cro protein binds to the operator sites (Lewin, 1987).

Some of the factors which influence phage replication include the concentration of calcium and magnesium in the growth medium, growth temperature and growth phase of the bacterial cells. Calcium must be present in the growth medium, although the mechanism whereby calcium affects phage growth in lactococci is unknown. Studies with *Lactobacillus casei* demonstrated that calcium ions are required for the

penetration of phage DNA into the host cell but they are not necessary for phage adsorption and intracellular multiplication (Watanabe and Takesue, 1972). The calcium requirement is claimed to be host specific, because three of eight phages tested had different calcium requirements when grown on different hosts (Potter, 1970). The calcium requirement is greater for phages growing on *L. cremoris* than on *L. lactis* (Lowrie and Pearce, 1971). On the other hand, calcium is required by the phage because a modified phage has the same calcium requirements when grown on *L. cremoris* and *L. lactis* (Lowrie and Pearce, 1971). Calcium may be required by both the phage and the host cell (Lawrence *et al.*, 1976).

In studies with phage lambda, development usually occurs in the first ten minutes after infection. This is followed by metabolic arrest in the host cell, reflected by a decreased oxygen uptake and decreased DNA and protein synthesis. Metabolic arrest is due to leakage of cations from the cell, which is reversible in the presence of high concentrations of magnesium ions (0.05 M). Magnesium ions may stabilize the cell membrane (Mathews, 1971).

An increase in lytic phage from *L. cremoris* and *L. lactis* has been observed when the temperature is increased from 40°C to 50°C. This has been attributed to a loss of plasmid mediated restrictive capabilities due to the environmental stress placed on the cell (Sanders and Klaenhammer, 1980). Phage infection is more efficient when stationary cells as opposed to exponentially growing cells of *L. cremoris* are used (Terzaghi and Terzaghi, 1978).

Lactococcal phages can be found almost anywhere in dairy plants including raw milk, pasteurized milk, whey, whey powder, skim milk, plant equipment, floors, walls, the air of rooms as well as on personnel and their clothes (Teuber and Lembke, 1983). Because phages are not destroyed by pasteurization, phage originating from wild-type lactococci found in raw milk can also be found in pasteurized milk (Heap and Lawrence, 1977).

Lysogenic starter strains are a major source of phage (Huggins and Sandine, 1977; Heap *et al.*, 1978; Terzaghi and Sandine, 1981). Lysogenic strains of lactococci were first demonstrated by Reiter (1949) who found three strains of lactococci which spontaneously release phage that infect and lyse an indicator strain. Huggins and Sandine (1977) examined 63 strains of lactococci and found that 38 of them carried phage. Terzaghi and Sandine (1981) reported that the majority of 45 strains of lactococci examined showed intact phages and that the remainder showed defective phage particles. It has been suggested that one lytic phage may develop from another by slight modification and thus present a source of "new" phage (Heap and Lawrence, 1976; Jarvis and Meyer, 1986). This has been confirmed by an electron microscopic heteroduplex study (which compares similarities between single strands of DNA) of three lactococcal phages (Jarvis and Meyer, 1986). DNA-DNA homology studies have demonstrated that phages of similar morphology are related (Jarvis, 1984, 1987). *L. lactis* and *L. cremoris* strains carrying a phage resistance plasmid (pTR2030) were resistant to all phages of one morphological group, which were isolated in both the United States and New Zealand (Jarvis and Klaenhammer, 1986). This suggests that lytic phages in cheese plants originate from only a few distinct phage types (Jarvis, 1987).

Historically, lactococci were susceptible to phage attack due to several factors: a) starter strains were often phage related, b) culture combinations were used over an extended period of time, and c) nonsterile pasteurized milk was used as a growth medium. Control of phage has been attempted by different methods such as the use of phage resistant starter strains or mutants, the rotation of phage unrelated strains and the use of phage inhibitory media. Three different types of phage resistance mechanisms have been identified: phage adsorption, restriction/modification (R/M) and abortive infection. Phage resistant mutants are unable to adsorb phage efficiently which is attributed to receptor site mutation causing a shift from phage sensitivity to

phage resistance (King *et al.*, 1983). Lactococci have different receptor sites which may be present in both the cell wall and cell membrane (Oram and Reiter, 1968), therefore a single mutation is unlikely to prevent adsorption of all phages. Blockage of phage adsorption to *L. lactis* ME2 has been linked to a 30 Mdal plasmid, pME0030 (Sanders and Klaenhammer, 1983). Many phage resistant mutants lack the acid producing activity of their parent strain and are therefore not suitable for commercial use. Mutations altering phage receptor sites may interfere with the proteinase and peptide transport systems (Lawrence, 1978).

The presence of R/M systems within the lactococci may explain the great variability and flexibility observed among lactococcal phages (Teuber and Lembke, 1983). The R/M system recognizes and degrades unmodified phage DNA. Those that escape destruction are modified during the process of lytic phage production and become immune to restriction by the host organism (McKay, 1985). R/M systems have been reported in *L. cremoris* strains C3, AM1, F and KH (Daly and Fitzgerald, 1982) and *L. lactis* strain ME2 (Sanders and Klaenhammer, 1984). In *L. cremoris* KH the R/M system has been linked to a 10 Mdal plasmid (Sanders and Klaenhammer, 1981) which may explain the rapid development of phage sensitive starter cultures.

Abortive infection is a phage resistance mechanism in which phage bursts are reduced or inhibited even though phage adsorption, DNA entry and initial stages of viral gene expression are normal (Sanders, 1988). Abortive infection is a plasmid mediated trait which has been identified in several strains of lactococci (Klaenhammer and Sanozky, 1985; Gauthier and Chopin, 1987). Plasmid mediated abortive infection in *L. cremoris* IL964 showed abortive infection in 97% of infected cells, while in the remaining 3%, a 72% decrease in burst size was observed (Gauthier and Chopin, 1987). The phage resistance plasmid pTR2030 (Klaenhammer and Sanozky, 1985) may be conjugally transferred to a variety of *L. lactis* and *L. cremoris* strains (Sanders *et al.*, 1986; Sing and Klaenhammer, 1986). Strains carrying this plasmid are

resistant to small isometric phages (Jarvis and Klaenhammer, 1987) and they may be used on a commercial level (Sanders, 1988). Some strains of lactococci have more than one phage defense mechanism. *L. cremoris* IL964 carries three phage resistance plasmids, two coding for R/M systems and one coding for abortive infection (Gautier and Chopin, 1987). *L. lactis* ME2 carries three different phage resistance plasmids, coding for interference of phage adsorption, R/M and abortive infection (Sanders and Klaenhammer, 1983, 1984; Klaenhammer and Sanozky, 1985). Plasmid encoded phage defense mechanisms may be incorporated into strains of lactococci using genetic engineering, which may lead to the construction of starter strains with improved phage resistance (Sanders, 1988).

The rotation of unrelated phage strains is also used to minimize phage infection of starter cultures. Carefully selected starter strains of different phage sensitivities are used in pairs or as multiple starters (Thomas *et al.*, 1977; Limsowtin *et al.*, 1977). When phage appears for a particular strain, that strain is removed from the blend and replaced with a phage unrelated strain. The advantage of using paired strains is that it minimizes the number of different phage types introduced into a cheese plant. Cheese starters have been propagated in the presence of whey obtained from the previous day's production (Thunell *et al.*, 1981) to make them more phage resistant. Cultures propagated in a cheese plant are less sensitive to phage than cultures propagated under laboratory conditions, which suggests that cells sensitive to phage are being continually eliminated and that the surviving strains are resistant to the phages present in the cheese plant (Jarvis, 1981).

Phage inhibitory media (PIM) contain phosphates and citrates which bind available calcium and thus interfere in the phage adsorption process. Bulk starter systems have been developed that combine neutralization of lactic acid with phage control (Richardson *et al.*, 1977; Sandine and Ayers, 1983). The most successful is

the patented Phase-4 medium (Morris, 1980). pH controlled PIM will be discussed in more detail in section 1.6

## 1.5 Acid injury

Lactococci produce lactic acid at the rate of more than 10% of their weight per minute (Sandine, 1985). Starter cultures lose activity and suffer damage upon prolonged incubation in milk, due to the exposure to high hydrogen ion concentrations (Lawrence et al., 1976). *L. cremoris* strains are generally more acid sensitive than strains of *L. lactis* (Thunell and Sandine, 1985). Bacteria establish and maintain an electrochemical gradient of hydrogen ions across their cytoplasmic membrane. This gradient consists of a transmembrane potential and a transmembrane pH gradient, which is negative and alkaline on the inside, giving rise to the proton motive force (Kashket *et al.*, 1980; Kashket, 1987). The proton motive force generated is the net result of the expulsion of hydrogen ions, catalyzed by the membrane $H^+$ ATPase complex; and their re-entry into the cell, mediated by a number of proton-linked transport systems (Kashket *et al.*, 1980). $H^+$ expulsion is also mediated by an electrogenic $H^+$/lactate symport, driven by the downhill efflux of lactic acid (Konings, 1985).

Growth of lactococci in milk causes a decrease in pH because of the lactic acid that is produced. At a certain pH, cell growth ceases but fermentation may continue until a pH of 4.2 to 4.4 is reached (Lawrence *et al.*, 1976). The cytoplasmic pH decreases as the external pH decreases, although it remains more alkaline than the external pH. Fermentation ceases when the pH of the cytoplasm is decreased to pH 5.7, i.e. at an approximate external pH of 4.5 (Kashket, 1987). Accumulation of acidic end products of fermentation results in acidification of the cytoplasm because the cells are unable to expell the $H^+$ fast enough. Incubation of *L. lactis* in broth culture adjusted to pH 5.0 results in a reversible reduction in the specific activities of cytoplasmic

enzymes, however, organisms shows an increased lag phase on subculture (Harvey, 1965).

Cell membrane damage and solute leakage was observed in *Streptococcus faecalis* (Marquis *et al.*, 1973). Below pH 5.0, magnesium ions were displaced from isolated cell walls and ribosomes by $H^+$. Increased permeablility of the cell membrane to $H^+$ may be responsible for reversible denaturation of internal structures (Marquis *et al.*, 1973) and vital cell components such as proteins (Lawrence *et al.*, 1976). Rapid lactose metabolism by cells of *L. lactis* in the stationary phase cause RNA breakdown and accelerated death. Increased lag times were observed for the survivors (Thomas and Batt, 1968, 1969). High concentrations of lactic acid increase the inward leak of $H^+$, so that the $H^+$ efflux is not fast enough to alkalinize the cytoplasm. This disrupts the pH gradient of the membrane. The cell dies when the cytoplasmic pH reaches a critical point (Kashket, 1987).

### 1.6 Bulk starter media

Milk and nonfat dry milk were commonly used as bulk starter media for lactococcal starters before the advent of pH controlled phage inhibitory media. Starter bacteria are more active and viable when grown in a pH controlled medium, because they are protected from acid damage. Phage infection is prevented by the addition of phophate and citrate salts, which bind calcium ions. Several pH controlled phage inhibitory media have been developed in the past decade and they are widely used in commercial cheese plants. Richardson *et al.* (1977) developed an externally pH controlled bulk starter system in which pH is maintained at 6.0 by injection of ammonium hydroxide directly into the bulk starter tank. The growth medium is composed of dilute whey which is fortified with phosphates and yeast extract. Cultures reached higher numbers, which allows for a 20 to 30% reduction of inoculum level, and starters may be stored without suffering acid damage because all of the lactose has

been utilized during growth (Richardson *et al.*, 1977). This system has been successfully used in manufacture of Cheddar, Monterey (Richardson *et al.*, 1977), cottage (Chen and Richardson, 1977), Italian and Swiss (Reddy and Richardson, 1977) cheeses. The composition of phage inhibitory media used in combination with pH control currently ranges from whey with added growth supplements to media which are completely milk based. External pH control allows for savings of up to two-thirds in media costs (Thunell and Sandine, 1985).

An alternative internal pH controlled phage inhibitory medium was developed which eliminates the need for pH electrodes, recorders and alkali injectors (Cogan and Daly, 1987). In 1979, Sandine and Ayers developed Phase-4, a bulk starter medium consisting of sweet whey, autolyzed yeast extract and phosphate-citrate buffers. The buffering ingredients are present in solid form and they are released at pH 5.1 to 5.2 (Morris, 1980). Internal pH controlled medium keeps the pH high enough to prevent acid injury of the cells but low enough to inhibit the growth of contaminating microorganisms (Mermelstein, 1982).

The titre of phages with low calcium requirement decreased in Phase-4 medium (Morris, 1980), however, some strains may reproduce in bacteria grown in phage inhibitory medium due to mutations (Morris, 1980). Phosphate and citrate salts in phage inhibitory medium may suppress growth of lactococci compared with their growth in nonfat milk (Wright and Richardson, 1982). Metabolic injury, ranging from 2 to 46% of the cells, has been observed in lactococci grown in phage inhibitory media of different formulations (Ledford and Speck, 1979). Reduced proteinase activity was observed in the presence of 2% phosphate. Proteinase plasmids may be lost when cells are grown in phage inhibitory medium (Ledford and Speck, 1979). When lactococci are grown in phosphated media, enlarged cells, atypical gram stains and a morphology indicative of disruption are observed. Where the pH is controlled between 6.0 and 6.2, concentrations of 1% phosphate may be used for external pH

controlled media without losing its phage inhibitory effects. At lower pH levels, higher concentrations of free soluble calcium are encountered, thus higher phosphate concentrations are required (Ausovanadom *et al.*, 1977).

Artificially pH controlled media are based on whey or milk and they contain added nutrients and buffering salts. However, milk concentrated by ultrafiltration displays a natural built-in buffering mechanism (Mistry and Kosikowski, 1986). Soft and semi-soft cheese can be manufactured from ultrafiltered (UF) milk retentates but they require highly active starter cultures to overcome the high buffering power and reach the desired pH for cheesemaking. This high buffering power could be exploited in the manufacture of bulk starter cultures. The advantage of manufacturing cheese with UF milk is that the whey proteins are included in the cheese (Glover, 1986), increasing the yield by 8 to 30% depending on the moisture content of the cheese (Hickey *et al.*, 1983).

Ultrafiltration is a pressure driven membrane separation process based on the pore size of the membrane. Large molecules such as fat and protein remain in the retentate; water, salts and lactose can pass through the membrane (Glover, 1986). Nonprotein nitrogen compounds, mainly urea and amino acids also pass through the membrane, 20 to 40% of the nonprotein nitrogen compounds remain in the retentate. Minerals such as calcium, magnesium, phosphate and citrate are partly bound to proteins and remain in the retentate, but other free minerals pass through the membrane. Fat soluble and protein bound vitamins also remain in the retentate. Up to 10% of the lactose may be retained (Glover, 1986). The main buffering components in milk are proteins, phosphate, citrate and bicarbonate (Jenness *et al.*, 1983). Casein, whey protein and minerals contribute 53.8%, 9.7% and 36.7%, respectively, to the total buffering capacity of a five-fold concentrated UF milk retentate (Srilaorkul *et al.*, 1989). Calcium and magnesium are present in several forms: as free ions, as soluble undissociated complexes with phosphate, citrate and casein, and as colloidal

phosphates associated with casein (Jenness *et al.*, 1983). The maximum buffering value at pH 5.1 for a five-fold concentrated retentate (18.5% total solids) is approximately six times greater than that reported for milk (Covacevich and Kosikowski, 1979).

As a result of the increased buffering capacity in UF retentates, starter organisms must produce larger amounts of lactic acid per unit change in pH, thus increasing the cheese manufacturing time. This can be partially overcome by increasing the inoculum size (Mistry and Kosikowski, 1985b). Maximum buffering in skim milk and UF milk retentates occurs between pH 5.1 and 5.3 (Mistry and Kosikowski, 1985a). As a result, pH is maintained above 5.0 longer in UF retentates than in milk, despite the continuing growth of starter bacteria. This minimizes cell injury and allows the cells to grow to higher numbers. The high buffering capacity therefore acts as a protective mechanism for starter cultures (Mistry and Kosikowski, 1986). Starter cultures grown in these naturally buffered retentates are more active and stable for longer periods of time than those grown in milk, and they are able to ferment UF retentates to the desired pH much quicker than conventional bulk starters (Mistry and Kosikowski, 1986).

Maximum cell numbers in skim milk and UF retentates occur around pH 5.2, below this level bacterial growth slows significantly but lactic acid production continues (Mistry and Kosikoski, 1985a). Growth is thus uncoupled from acid production (Turner and Thomas, 1975; Hickey *et al.*, 1983). Lactose metabolism in milk is inhibited when the pH reaches 4.2 to 4.4, which corresponds to about 0.7% lactic acid. However, in a 5.8-fold concentrated UF retentate, starter cells are stable and do not suffer damage, even in the presence of 1.8% lactic acid (Mistry and Kosikowski, 1985a).

## 1.7 Freezing and storage

Freezing is a useful method for preserving, maintaining and storing starter cultures for extended periods of time. The starter may be frozen directly or it may be concentrated for use in bulk and direct-to-vat inoculations. Much research has been done to determine the optimum conditions necessary to achieve maximum cell yield with high acid producing activity and ability to survive freezing and low temperature storage. The metabolic activity of the culture must be maintained throughout its preparation and storage to ensure product uniformity and quality. Bacterial strains differ in their susceptibility to freezing and thawing, which is influenced by growth medium and conditions of freezing, storing and thawing. Some general principles with respect to freeze injury suffered by bacterial cells have been observed: (a) survival decreases with extended storage (Moss and Speck, 1963; Gibson *et al.*, 1965); (b) death rate is reduced at lower storage temperatures (Gibson *et al.*, 1965; Bauman and Reinbold, 1966); (c) death rate is reduced when cultures are neutralized before freezing (Peebles *et al.*, 1969; Lloyd and Pont, 1973; Gilliland and Speck, 1974a; Efstathiou *et al.*, 1975); and (d) freeze injury is minimized in the presence of cryoprotective agents (Lamprech and Foster, 1963; Bauman and Reinbold, 1966; Gibson *et al.*, 1966; Stadhouders *et al.*, 1971; Lloyd, 1975).

Maximum survival occurs under conditions of rapid freezing accompanied by fast thawing (Bauman and Reinbold, 1966; Gibson *et al.*, 1966; Lloyd, 1975). Freeze injury suffered by bacteria is due to cellular damage which is brought about by elevated solute concentrations causing cell dehydration and by ice crystals causing membrane destruction (Litvan, 1972). Under conditions of rapid cooling, the cell becomes dehydrated because water is released at a faster rate than membrane permeability allows, causing leakage of cellular components through small holes in the cell membrane or total destruction of the membrane (Ray and Speck, 1973). Growing cultures are most susceptible to cold shock (Ingraham *et al.*, 1983). Smaller ice

crystals are formed during rapid freezing resulting in less cellular damage than during slow freezing (Bauman and Reinbold, 1966; Litvan, 1972). Small intracellular ice crystals may grow in size by recrystallization at mild freezing temperatures and during thawing. Ice crystals formed during freezing and recrystallization may cause damage to the cell membrane (Ray and Speck, 1973). After freezing and thawing, cell cultures contain uninjured, reversibly and nonreversibly injured as well as dead cells. Some cultures display an extended lag phase upon thawing, this may be the result of recovery of injured cells prior to cell multiplication (Thunell *et al.*, 1984).

Freezing and storage of lactococci in liquid nitrogen at -196°C is a very effective means of storage, regardless of whether the cells have been cultured in broth or milk based media (Lloyd, 1975). However, this is an expensive method of storage and only small quantities can be frozen at any one time (Lawrence *et al.*, 1976). Concentrated cultures of lactococci remain viable without loss of activity for periods up to 231 days at -196°C (Peebles *et al.*, 1969). Survival of lactococci in concentrated cultures was 42% at -20°C, 93 to 99% at -40°C and 100% at -110°C (Lloyd and Pont, 1973). Although freezing and storage in liquid nitrogen provides the best conditions for cell viability (Cowman and Speck, 1963; Bauman and Reinbold, 1966; Gibson *et al.*, 1966; Stadhouders *et al.*, 1971; Lloyd, 1975), storage facilities at temperatures between -20°C and -40°C are more readily available.

While some strains of lactococci can be effectively maintained when stored at -20°C to -30°C, there is variation among strains. Some strains do not survive well when frozen at these temperatures (Lamprech and Foster, 1963; Accolas and Auclair, 1967; Bergere, 1968; Gilliland and Speck, 1974a; Lloyd, 1975). Death of bacteria, metabolic injury and loss of proteinase activity were observed when *L. lactis* was frozen and stored at -20°C (Cowman and Speck, 1963; Moss and Speck, 1963), variable results were obtained at temperatures of -40°C (Gibson *et al.*, 1965, 1966; Stadhouders *et al.*, 1971; Lloyd, 1975; Leach and Sandine, 1976). Most cultures can

be stored for short times at -20°C to -40°C, however, lower temperatures should be used for longer term storage. Marked decreases in cell viability and activity are observed at storage temperatures at -20°C and above. Addition of cryoprotective agents is often necessary at these temperatures (Bauman and Reinbold, 1966; Gibson *et al.*, 1966; Lloyd, 1975). Even though starter concentrates stored at -40°C have a slightly lower percentage of surviving cells compared with cells stored in liquid nitrogen, the difference is unlikely to be significant when they are used to inoculate bulk starter tanks (Lawrence *et al.*, 1976).

Maximum cell numbers in milk or broth are obtained when lactococci are cultured under controlled pH levels of 6.0 to 6.5 (Gilliland and Speck, 1974a; Efstathiou *et al.*, 1975). Cells survive freezing and storage at -20°C much better when frozen at pH 7 compared with pH 5 (Lamprech and Foster, 1963), or at -17°C at pH 6.0 than without pH control (Gilliland and Speck, 1974a). Reduction in cell numbers and activity were minimized when cultures were neutralized to pH 6.5 with NaOH prior to freezing at -20°C to -40°C (Stadhouders *et al.*, 1969). When $NH_4OH$ is used in place of NaOH as a neutralizer, the maximum cell numbers increased two-fold (Peebles *et al.*, 1969; Lloyd and Pont, 1973; Gilliland and Speck, 1974a; Efstathiou *et al.*, 1975). Reduced proteinase activity is observed when cultures are neutralized with $NH_4OH$ (Peebles *et al.*, 1969; Gilliland and Speck, 1974a), but cells are more resistant to freezing than cells neutralized with NaOH (Efstathiou *et al.*, 1975). Ammonia may be stimulatory to the cultures because it may serve as a nitrogen source for the formation of glutamic acid or glutamine (Peebles *et al.*, 1969). Free ammonia crosses the cell membrane by simple diffusion. Once inside the cell membrane some of the intracellular acid may be neutralized, thus raising the intracellular pH more rapidly than NaOH (Peebles *et al.*, 1969). Reduced cell numbers and activity are observed when cultures are neutralized with NaOH instead of $NH_4OH$. This may be attributed to inhibitory effects of the sodium ion (Peebles *et al.*, 1969).

Accolas and Auclair (1967) and Bergere (1968) reported that cells frozen in the late stages of exponential growth retain optimum activity. Moss and Speck (1963) and Lamprech and Foster (1963), on the other hand, suggested that cells in the stationary growth phase survive freezing better than cells in the exponential phase.

Cryoprotective agents have been added to starter cultures in attempts to improve their survival during freezing and frozen storage. Cryoprotectants inhibit the formation of intracellular or extracellular ice crystals by binding water. They influence the amount of dissolved salt and alter the properties of the bacterial membrane (Stadhouders *et al.*, 1969). Even though glycerol is an effective cryoprotective agent, its use in dairy starter cultures is not acceptable because it is not a constituent of milk. Lactose is preferred as a cryoprotective agent (Stadhouders *et al.*, 1971). Variable results have been reported on the ability of glycerol and lactose to provide protection during frozen storage. Some strains are adequately protected while others are not (Stadhouders *et al.*, 1971; Efstathiou *et al.*, 1975). Strains of *L. cremoris* and *L. lactis* were protected in the presence of glycerol when stored at -23.3°C, but they had lower rates of acid development during incubation after thawing (Gibson *et al.*, 1966). Freezing and storage of lactococci at -37°C in the presence of 10% glycerol showed variable results, while the addition of 7.5% lactose allowed storage for up to 3 months without loss of activity (Stadhouders *et al.*, 1971). However, lactose was found to be ineffective by others (Efstathiou *et al.*, 1975). Lactococcal starter cultures stored in Phase-4 medium retained their activity and viability in the absence of glycerol at -40°C, and in the presence of glycerol at -20°C, for up to 3 months (Thunell *et al.*, 1984). Added glycerol protects cultures from freeze injury at pH 5.0 but offers no added benefit at pH 7.0 (Stadhouders *et al.*, 1971). Storage temperature, however, is the single most important factor in maintaining starter activity (Bauman and Reinbold, 1966). Optimum storage

temperature for starter cultures without cryoprotective agents is -40°C and below (Stadhouders *et al.*, 1969, 1971).

The fatty acid composition of the cells and the amount of capsular material associated with lactococci appear to be closely related to survival of cells during freezing at -17°C, although contrasting results have been obtained. Gilliland and Speck (1974b) reported that the percentage of survivors decreased as the percentage of oleic acid in the cellular lipids of different cultures increased. No correlation between survival and cellular content of oleic acid was found when the cultures were grown at pH 6.0. Goldberg and Eschar (1977), on the other hand, reported an increase in percentage survival as the percentage of oleic acid was increased. An increase in the ratio of unsaturated to saturated fatty acids was also linked to increased survival rates. This is important in maintaining membrane fluidity and thus preventing cell death during freezing (Goldberg and Eschar, 1977). Stability of the membrane during freezing is most likely influenced by the overall balance of fatty acids as opposed to a single fatty acid (Gilliland and Speck, 1974b).

Capsules may provide some protection to the cells from injury due to freezing. Cultures containing larger amounts of capsular material were more resistant to injury than cultures with lower amounts (Gilliland and Speck, 1974b). The capsular material may provide structural stability to the cells so that they can better withstand the stress of freezing. Cultures grown at pH 6.0 did not contain larger amounts of capsular material than cultures grown without pH control (Gilliland and Speck, 1974b).

## 1.8 Discussion and conclusion

Much research has been done in the area of starter cultures and their maintenance, especially with respect to lactose metabolism and proteolytic activity because these are the characteristics of primary importance in cheese manufacture.

Cultures must possess the ability to ferment lactose efficiently and break down milk proteins to amino acids and peptides for growth without producing bitter peptides.

Starter culture activity can be impaired by exposure of the cultures to low pH, freezing temperatures and bacteriophage. Bulk starter media such as ICM and UF milk retentates have a high buffering capacity which protects starter cultures from exposure to low pH. ICM also has phage inhibitory properties but phage susceptibility in UF retentates has not been adressed in the literature. This study will investigate the effects of different bulk culture media on proteolytic activity and/or lactose metabolism in the presence of bacteriophage, low pH and at a temperature of -70°C.

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# 2. COMPARATIVE EVALUATION OF BULK STARTER SUBSTRATES ON ACTIVITY AND STORAGE OF TWO COMMERCIAL STARTER STRAINS [1]

## 2.1 Introduction

Traditionally, reconstituted skim milk (RSM) was used to prepare bulk starters because of its low cost, elimination of seasonal variation in composition and near absence of inhibitory substances and undesirable organisms (Davis, 1965; Pearce and Brice, 1973). The activity of starters in 10% RSM was reduced during prolonged storage at pH below 5.0 (Harvey, 1965). In 1977, a bulk culture system for Cheddar cheese manufacture was developed using external pH control with a whey-based bacteriophage inhibitory medium. A pH of 6.0 is maintained by injections of anhydrous ammonia directly into the starter tank (Richardson *et al.*, 1977). In 1979, an internal pH controlled medium was developed, which was composed of sweet whey, yeast extract and phosphate and citrate buffers. The medium is buffered so that the pH remains above 5.1-5.2, thus preventing acid injury of the bacteria (Sandine and Ayers, 1983).

Because the pH is maintained above 5.0 in both the external and internal pH controlled media, starter organisms grow to higher numbers and maintain their activity over a longer period of time compared with organisms grown in RSM. More recently, the growth and acid production of starter organisms in ultrafiltered (UF) milk retentates have been investigated (Hickey *et al.*, 1983; Mistry and Kosikowski, 1985). UF retentates have a natural buffering system due to the increased mineral and

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milk protein contents. The high buffering capacity of UF milk is regarded as a drawback for manufacture of cheese. Starter cultures must be very active and produce large amounts of lactic acid to overcome the natural buffering capacity so that the desired pH for cheesemaking is reached (Covacevich and Kosikowski, 1979). However, the natural buffering capacity of UF skim milk could be exploited for the production and maintenance of active bulk starter cultures during storage. UF retentates have the potential to provide a protective environment for starter organisms by minimizing acid injury, which allows the starter to grow to greater numbers (Mistry and Kosikowski, 1986). Even when the pH drops below 5.0, the activity of the starter culture is not impaired.

It is common practice in some smaller dairy industries to prepare a bulk starter once a week, to store it at refrigeration temperature and to use it for up to 5 days. The objectives of this study were to compare the effects of storage at 2°C on the activity of two strains of lactic acid bacteria in reconstituted skim milk, UF retentates and a commercial internal pH controlled medium.

## 2.2 Materials and methods

### 2.2.1 Culture media

Commercial skim milk powder and commercially pasteurized skim milk (high temperature, short time) were tested for the presence of antibiotic substances using the *Bacillus stearothermophilus* disk assay (Richardson, 1985). The skim milk powder was reconstituted with distilled water to contain 10%, 20% and 24% of dry matter. RSM media were sterilized by steaming on two successive days at 90°C for 30 min and stored overnight at 2°C to eliminate spore forming bacteria. Pasteurized skim milk was concentrated at 50°C to volume concentration factors of 2:1, 3:1, 4:1 and 5:1

(subsequently referred to as R2:1, R3:1, R4:1 and R5:1) in a laboratory scale ultrafiltration unit (DDS-20 Lab Module, DDS, Nakskov, Denmark) using a 0.018 $m^2$ membrane (GR60-PP) with a 25,000 molecular weight cut-off. The retentates were divided into 200 mL aliquots in 250 mL Nalgene polypropylene bottles, steamed once at 90°C for 30 min and frozen at -20°C until required for use in experiments. The samples were thawed in a microwave oven using the defrost cycle. An internal pH controlled medium (ICM), consisting of a special blend of phosphate buffers, grade A sweet whey, and autolyzed yeast extract (prepared for Nordica International formula 55-0003 by UFL Foods, Edmonton, Canada) was used as a commercial reference medium.

### 2.2.2 Composition of milk media

Composition of the RSM media and retentates was determined by standard methods for milk analysis (Richardson, 1985). Total solids was determined by evaporation on a steam bath for 30 min, followed by drying in a forced air oven at 100°C for 3.5 h. Ash was determined by ignition of a dried sample in a muffle furnace at 550°C. Fat content was determined by the Mojonnier method. Total protein was determined by the macroKjeldahl method using 6.38 as the conversion factor for nitrogen to protein. Lactose concentration was determined by HPLC, using 5 and 10% lactose solutions as standards (Euber and Brunner, 1979).

### 2.2.3 Measurement of buffering capacity

Duplicate 20 mL samples of selected media were tempered to room temperature and titrated by successive additions of 50 µl of 1 N HCl to the 10% RSM and 100 µl additions of 1 N HCl to all other media at 15 min intervals, while being constantly stirred on a magnetic stirrer. The pH was measured until pH 4.4 was reached.

Titration curves were drawn, plotting the amount of 1 N HCl vs pH. The buffer index was calculated using the following equation:

$$dB/dpH = \frac{(\text{mL acid or base})(\text{normality factor})}{(\text{volume of sample})(\text{change in pH})}$$

Buffering capacity curves were obtained by plotting the buffer index against pH (Van Slyke, 1922).

### 2.2.4 Preparation of starter organisms in bulk culture media

Commercial strains of *Lactococcus cremoris* strains 103, 108, 202, 205 and 208 were obtained from a local dairy plant and screened for acid sensitive and acid insensitive strains. All strains were purified, suspended in sterile glycerol citrate on sterile filter strips, sealed in plastic pouches and stored at -70°C. The preserved strains were reconstituted for use by transferring a filter strip to 10 mL of Elliker broth (Difco Laboratories Inc., Detroit, MI) and incubating at 32°C for 16 h. Strains were subcultured twice in 10% RSM and incubated at 32°C for 16 h. RSM cultures were subsequently inoculated (1.5%, v/v) into 100 ml of the experimental bulk starter media, mixed thoroughly by shaking and 10 mL was aseptically dispensed in sterile test tubes. The tubes were incubated at 32°C for 16 h and stored at 2°C for up to 7 days.

### 2.2.5 Measurement of starter activity

The activity of the test strains was screened in 10% RSM. Strains 108 and 205 were selected for further study based on their sensitivity and insensitivity to

acidity, respectively. On day 0 and after 2, 5 (and 7) days of storage, the activity of the two strains stored in each medium was evaluated by inoculating 0.75 mL of the bulk culture into 50 mL of tempered 10% RSM, mixing and dividing it into 5 mL aliquots in 10 sterile test tubes. These inoculated samples were incubated at 32°C and one of the tubes was removed at hourly intervals to measure pH, using a Fisher Thin Electrode with a Fisher Accumet model 230 pH meter. pH determinations were continued until pH 4.6 was reached or for a maximum of 10 h. Bacterial numbers were monitored on APT (Difco) agar plates. The pH of each medium was monitored during the 5 day storage period. The entire experiment comparing the effect of the six substrates was repeated three times. A computerized statistical package (BMDP2V, BMDP Statistical Software, 1983. University of California Press, Berkeley, CA) was used to determine the effects of the bulk culture media on the activity of the cultures, excluding the 10% RSM. Analyses were done separately for strain 108 and 205 based on the time taken to reduce the pH to 4.6 or 5.2. Scheffe's test was used for the ranking and selection of the bulk culture media (Brook and Arnold, 1985; Gibbons *et al.*, 1977).

### 2.2.6 Agglutination behaviour of starter cultures in raw milk and 10% RSM

Agglutinin titers of the two strains were determined according to the method of Emmons *et al.* (1965). Raw, whole milk obtained from the University of Alberta Dairy Research Station was skimmed by centrifugation at 25,000 x g for 10 min. Test strains 108 and 205 were inoculated (1.5%) into 100 mL of raw skim milk or 10% RSM and dispensed into a series of three 25 x 150 mm test tubes. The tubes were incubated at 32°C. pH measurements were made at the top, middle (6.5 cm depth) and bottom (13 cm depth) of each series of tubes at hourly intervals until pH 4.6 was reached.

## 2.3 Results

The preliminary study showed that *L. cremoris* strain 108 was relatively acid tolerant in a 10% RSM bulk culture at pH 4.5 for up to 5 days. In contrast, strains 103, 205 and 208 lost activity with increasing storage time, strain 205 was the most sensitive to acid. Strain 202 showed progressively slower and more variable acid production during storage. Strains 108 and 205 were chosen for further studies.

When strains 108 and 205 were grown and stored in R3:1, R4:1 and R5:1 and inoculated into 10% RSM at 32°C, the pH achieved after 6 h incubation was very similar. Based on these data, further studies were undertaken using R2:1 and R3:1 as media for bulk cultures. The viscosity of R4:1 and R5:1 media posed problems in sterilization of the UF concentrate and in the distribution of the starter organisms after inoculation.

The proximate analyses of the bulk starter RSM media and selected UF retentates are shown in Table 2.1. In R2:1 and 20% RSM media, the protein content was almost the same, but total solids differed by almost 7 g/100 mL because of the proportionally reduced lactose content of UF milk. In R3:1 and 24% RSM media, however, the difference in total solids content was approximately 7 g/100 mL, and protein content differed by >1 g/100 mL.

The maximum buffering capacities for the RSM media (Fig. 2.1) and UF retentates (data not shown) are between pH 4.7 and 5.1, whereas the maximum buffering capacity for ICM is at pH 5.5 (Fig. 2.2). The buffering capacities of the 20% RSM and R2:1 are twice that of 10% RSM, and those for 24% RSM and R3:1 are almost four times greater than 10% RSM. However, the ICM had a buffering capacity fifteen-fold greater than that of 10% RSM at the maximum buffering value (Fig. 2.2). Activity of the test cultures stored in the bulk starter media was measured by determining the time for the pH of the freshly inoculated 10% RSM incubated at 32°C

Table 2.1 Proximate composition of reconstituted skim milk and UF bulk culture media.

| SUBSTRATE | TOTAL SOLIDS% | FAT% | PROTEIN % | LACTOSE % | ASH % |
|---|---|---|---|---|---|
| 10% RSM | 10.08 | 0.08 | 3.44 | 5.2 | 0.78 |
| 20% RSM | 20.13 | 0.14 | 6.78 | 10.0 | 1.50 |
| 24% RSM | 24.00 | 0.17 | 8.19 | 12.0 | 1.83 |
| R2:1 | 13.29 | 0.23 | 6.44 | 4.9 | 0.94 |
| R3:1 | 16.81 | 0.26 | 9.27 | 4.6 | 1.23 |

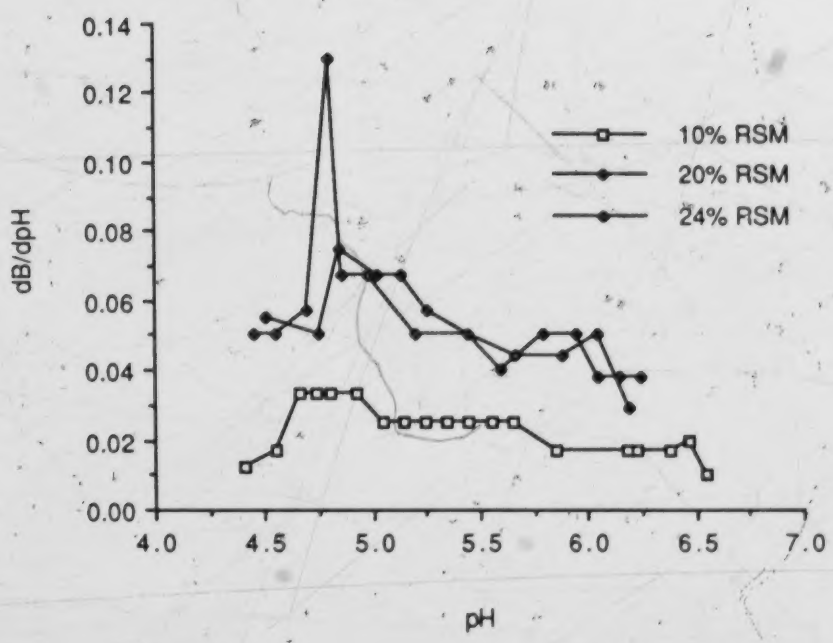


Fig 2.1 Buffering capacity (dB/dpH) of reconstituted milk media (10%, 20% and 24%) determined as a function of change in pH by titration with 1 N HCl.

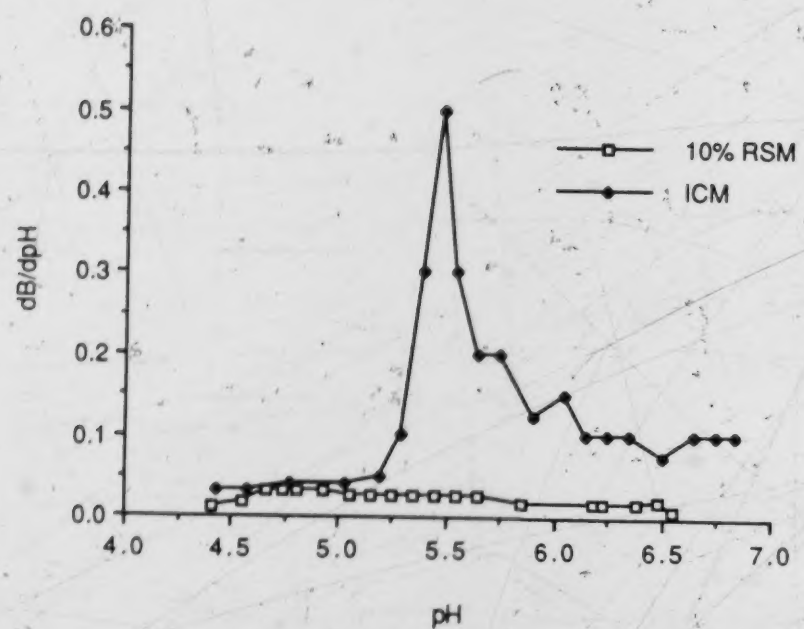


Fig 2.2 Buffering capacity (dB/dpH) of ICM compared with 10% RSM determined as a function of change in pH by titration with 1 N HCl.

to reach 5.2 and 4.6. Strain 108 showed less acid injury than strain 205 (Fig. 2.3 and 2.4), but some loss of activity occurred after storage in 10% RSM for 5 days. An additional 1.5 h of incubation was required for the culture to achieve pH 5.2 and 4.6 on day 5 than on day 0 (Fig. 2.3). In contrast, storage of strain 205 in 10% RSM at 2°C for 5 days caused severe injury to the culture, resulting in a lag phase of at least 10 h. The 20% and 24% RSM and the R2:1 and R3:1 retentates protected the strains from acid injury (Fig. 2.5).

It was considered that the apparent increase in the lag phase observed in strain 205 in 10% RSM may be due to agglutination or settling of the starter cultures in the culture tube. Agglutination determined in undiluted raw milk whey gave positive results for both strains. No agglutination was observed in 50% whey or in whey prepared from 10% RSM at 50% dilution, and variable results were obtained with undiluted whey obtained from 10% RSM. When the pH of the inoculated raw and 10% RSM in culture tubes was measured at different levels in the tube on day 0 and day 5, the pH was the same at all three locations. This indicates that the increased lag time was not due to clumping of the organisms.

A multiple analysis of variance (Table 2.2) with repeated measurements of the data (excluding 10% RSM) showed a significant effect ($P<0.05$) attributable to the effect of the growth medium on the maintenance of activity of both starter strains stored at 2°C. No significant effect ($P>0.05$) was attributable to replicates or time of storage. There were significant statistical differences in the maintenance of activity between the two strains in the corresponding media (Table 2.2). Strain 205 maintained its activity significantly ($P<0.05$) better in R3:1 than in 20% and 24% RSM, R2:1 or ICM, with no significant difference among these four media. In contrast, strain 108 showed a significant difference ($P<0.05$) between 24% RSM and R2:1, but no significant difference among 24% RSM, 20% RSM, R3:1 and ICM. No significant difference among 20% RSM, R2:1, R3:1 and ICM was noted for strain 108 at either of

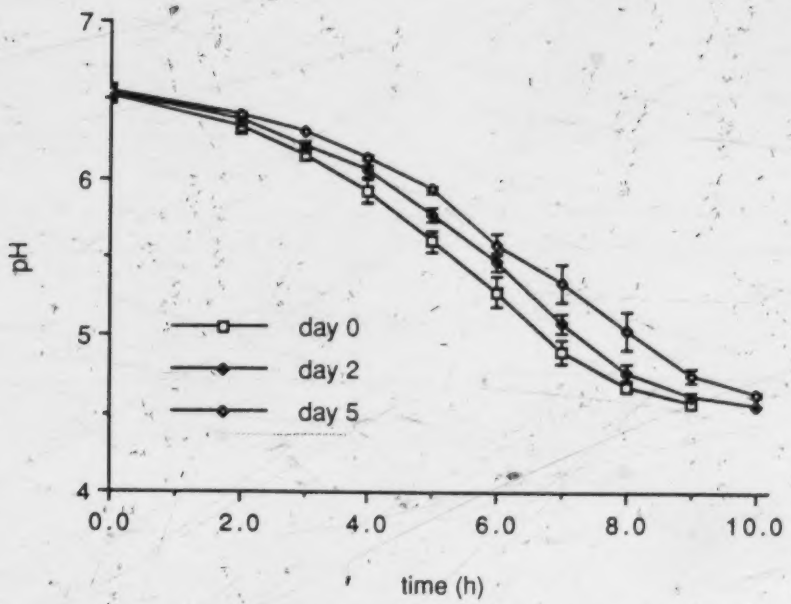


Fig 2.3 Effect of growth and storage in 10% RSM at 2°C for 5 days on activity of *L. cremoris* strain 108.

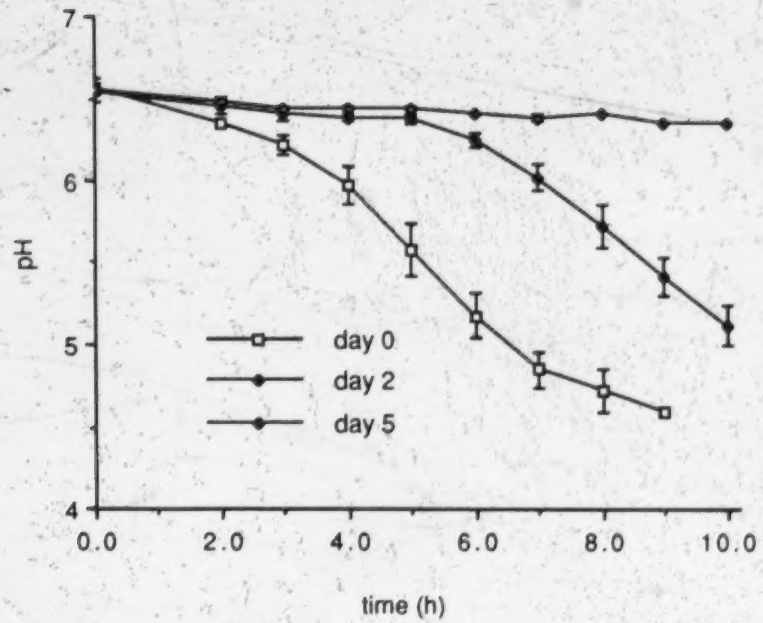


Fig 2.4 Effect of growth and storage in 10% RSM at 2°C for 5 days on activity of *L. cremoris* strain 205.

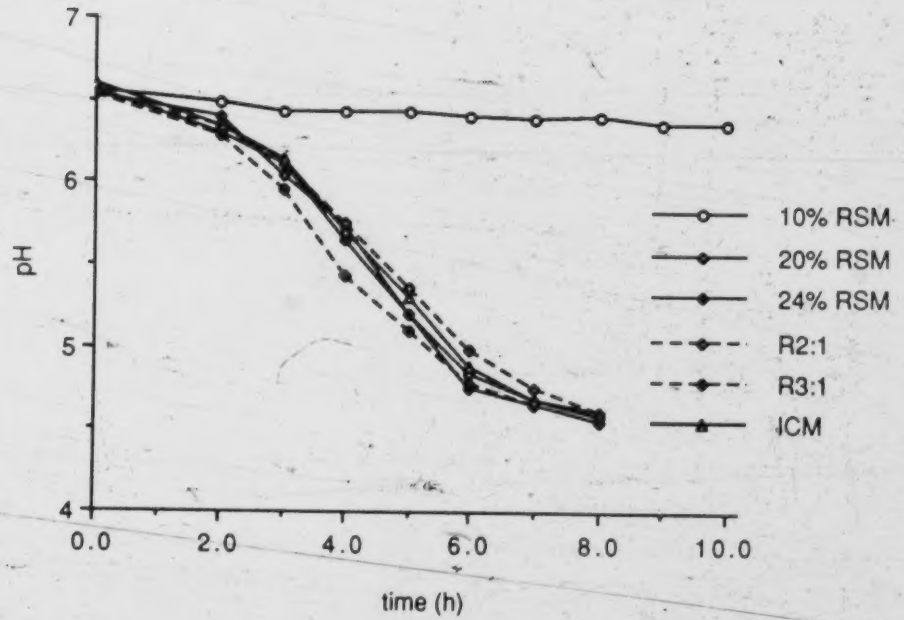


Fig.2.5 Effect of growth and storage in different bulk media at 2°C for 5 days on activity of *L. cremoris* strain 205.

Table 2.2 Rank order of mean time for *L. cremoris* strains 108 and 205 to achieve pH 4.6 in 10% RSM after storage in different media and significance of difference among means determined at the 95% confidence level using Scheffe's test*.

| STRAIN | Rank order of substrates (shortest to longest time to achieve pH 4.6) | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| 108 | 24% RSM | R3:1 | 20% RSM | ICM | R2:1 |
| 205 | R3:1 | 20% RSM | R2:1 | 24% RSM | ICM |

*Substrates underlined with an unbroken line are not statistically different.

the pH levels.

The pH of cultures during storage and the amount of lactic acid produced by the strains in each medium are presented in Table 2.3. The pH of the ICM during storage was above 5.0, whereas the pH for all other media was below 5.0. Viable counts of strain 205 during storage in each medium for 5 days is shown in Table 2.4. Die off of the cells was minimal, even in the cultures grown and stored in 10% RSM. Furthermore, the cells were not injured for growth on APT agar. The results show that almost five times as much acid was produced by the cultures growing for 16 h in ICM than in 10% RSM, and at least twice as much acid was produced in the ICM compared with the 24% RSM or R3:1 (Table 2.3).

## 2.4 Discussion

pH controlled bulk starter media were developed as alternatives to growth and storage in RSM, because cells remain more active and display less lag time when inoculated into milk for cheesemaking (Ustunol *et al.*, 1986). Cultures grown in ICM have to produce large amounts of lactic acid to overcome the high buffering capacity of the medium, as a result they would seldom be exposed to pH below 5.0. The buffering capacity of the UF retentates in this study was comparable to those reported by Srilaorkul *et al.* (1988), however, the buffering capacities were considerably less than that of ICM. At maximum cell growth the pH of the media with increased milk solids did not fall below 4.7 to 4.8, whereas the final pH in 10% RSM was 4.3. There is either a critical point at which acid injury occurs between pH 4.3 and 4.7, or the increased concentration of milk solids protects the cells from acid injury. It was expected that cell numbers would be higher in ICM than in the media with increased milk solids, because of the greater amount of lactic acid produced (Table 2.2). However, viable cell numbers were similar in each medium, indicating that lactic acid production was uncoupled from growth of the bacteria or the cultures were more active

Table 2.3 Final pH and amount of acidity produced during growth of *L. cremoris* strain 108 and 205 in bulk culture media at 32°C for 16h*.

| | STRAIN 108 | | STRAIN 205 | |
|---|---|---|---|---|
| STORAGE SUBSTRATE | final pH | lactic acid (µmol/mL) | final pH | lactic acid (µmol/mL) |
| 10% RSM | 4.35 | 49 | 4.30 | 50 |
| 20% RSM | 4.65 | 86 | 4.70 | 84 |
| 24% RSM | 4.80 | 78 | 4.80 | 78 |
| R2:1 | 4.60 | 89 | 4.60 | 89 |
| R3:1 | 4.70 | 108 | 4.70 | 108 |
| ICM | 5.40 | 225 | 5.35 | 235 |

*Mean of three replicate studies

Table 2.4 $Log_{10}$ of colony forming units (CFU)/mL* for *L. cremoris* strain 205 grown at 32°C for 16 h and stored at 2°C in different bulk culture media.

| SUBSTRATE | DAY 0 | DAY 2 | DAY 5 |
|---|---|---|---|
| 10% RSM | 8.50 | 8.60 | 8.02 |
| 20% RSM | 8.96 | 8.53 | 9.09 |
| 24% RSM | 8.88 | 8.58 | 8.61 |
| R2:1 | 8.95 | 8.66 | 8.43 |
| R3:1 | 8.96 | 8.61 | 8.83 |
| ICM | 8.79 | 8.65 | 8.91 |

*Geometric $log_{10}$ mean count of three replicate studies

cultures, but this was considered to be only marginal after 5 days of storage at 2°C.

Times to achieve pH values of 5.2 and 4.6 in 10% RSM were chosen as indicators of starter activity because pH 5.2 is the acidity required in Cheddar cheese manufacture before milling, and pH 4.6 is the point at which the curd is cut in cottage cheese manufacture (Kosikowski, 1966). Based on the acidity data for the starter strains used in this study, ICM could be replaced with either 24% RSM or R3:1 as a bulk culture medium to achieve optimum activity of the starter culture when subcultured into milk for cheese manufacture. This could be of importance to the dairy industry because skim milk powder and fluid milk are more readily available, and special starter media or neutralizing chemicals would not be required. Limitations to using skim milk powder to increase the total solids content are its microbial quality and its viscosity due to the high total solids content. Skim milk powders may be contaminated with sporeforming bacteria which could germinate and grow after steaming. Hence, skim milk powder intended for use as a bulk starter medium would need to be specially selected with low spore counts and absence of antibiotics.

UF retentates could also be used as alternatives to ICM as bulk starter media because the milk can be pasteurized before concentration and temperatures during UF can be adjusted to suppress bacterial growth. Furthermore, viscosity does not pose a problem up to a concentration factor of 3 (R3:1). UF retentates have been studied for use as bulk starter media (Mistry and Kosikowski, 1986). Cultures grown in a UF retentate (concentration factor of 4) was stored for an extra 8 h at 22°C beyond its 12 h incubation time. It was reported that there was no damage when the pH dropped below 5.0, so that a bulk culture could be prepared once daily and used for the whole day. Our results showed that 24% RSM and R3:1 had the same advantages as pH controlled media. The cultures remained stable for prolonged periods of time after incubation, they retained their activity and did not display an increased lag time when inoculated into cheese milk. Therefore, we consider that both 24% RSM and R3:1 could

be used as media for bulk starters. Although, this observation should be studied for a broader spectrum of starter strains, the results are promising because strain 205 is highly sensitive to acid.

This study illustrates that for smaller dairy industries, bulk cultures with 24% milk solids or 3:1 UF retentates could be made once a week, stored at refrigeration temperature and used for up to 5 days without impairment of starter activity or decrease in cell numbers. The same protection against acid injury was achieved when the buffering capacity was increased by increasing the skim milk solids content or by concentrating milk by UF. An aspect of this system that warrants study is the phage susceptibility of *L. cremoris* in UF retentates and media with increased milk solids compared with internal or external pH controlled media.

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# 3. STORAGE STABILITY AND BACTERIOPHAGE SUSCEPTIBILITY OF COMMERCIAL STARTER CULTURES IN BULK CULTURE MEDIA

## 3.1 Introduction

The presence of phage in milk fermentations is of major concern to the dairy industry because phage can attack and destroy starter cultures, leading to vast economic losses. Lysogenic starter strains have been implicated as a major source of phage (Huggins and Sandine, 1977; Heap *et al.*, 1978; Terzaghi and Sandine, 1981), therefore it is important to ensure that strains are screened for prophage. Lysogenic strains can release their prophage spontaneously or by induction. Induction with UV light may cause an alteration in the prophage structure in such a way that the repressor protein can no longer bind to the operator site or cause a change in the repressor protein properties after induction, hence preventing binding (Mathews, 1971).

External pH controlled bulk starter culture media (Richardson *et al.*, 1977) and internal pH controlled media (Sandine and Ayers, 1983) offer protection against phage in addition to protection from exposure to low pH. Phosphates and citrates present in these bulk culture media bind the available calcium and thus interfere with phage adsorption. Highly active starter cultures can be obtained when UF retentates are used as bulk culture media because of their naturally built-in buffering power (Mistry and Kosikowski, 1985, 1986). However, the question of phage susceptibility of starter cultures in UF retentates has not been adressed.

The objectives of this study were to determine a) the activity of *L. cremoris* strains 103, 108, 202, 205, 208, 777, 804 and 888 after 5 days of storage at 2°C in four bulk culture media: 10% reconstituted skim milk (RSM), 24% RSM, ultrafiltered milk (UF) retentate (R3:1) and internal pH control media (ICM), b) if

these strains carry prophage and their phage sensitivity to characterized phage strains, and c) the phage inhibitory properties of the four bulk culture media.

## 3.2 Materials and Methods

### 3.2.1 Bacterial strains

Frozen commercial strains *Lactococcus lactis* ssp. *cremoris* (*L. cremoris*) 103, 108, 202, 205, 208, 777, 804 and 888 (prepared as previously described in section 2.2.4) were used in this study. Two homologous bacteriophage strains, phage 202 and 804 obtained from Nordica International Inc., Sioux Falls, S. D., were stored at 2°C.

### 3.2.2 Storage stability of starter cultures at 2°C

Activity of the strains 103, 108, 202, 205, 208 and 777 was determined before and after 5 days of storage at 2°C in 10% RSM, 24% RSM, UF retentate R3:1 and ICM by measuring the change in percent titratable acidity (Δ%TA) after incubation at 30°C for 8 h when grown in 10% RSM. Bacterial numbers were monitored on APT (Difco Laboratories Inc., Detroit, MI) agar plates and the pH of each medium was measured before and after the 5-day storage period. These trials were done in triplicate.

### 3.2.3 Activity of starter cultures

The activity of starter cultures was determined by measuring the change in titratable acidity (TA) after incubation at 30°C for 8 h. A 9 mL amount of distilled water was added to 9 mL of inoculated sample in a 50 mL Erlenmeyer flask and 50 µL of 1% phenolphthalein (Fisher Scientific Company, Fair Lawn, N. J.) was added to 10% RSM samples, 100 µL to 24% RSM and R3:1 and 150 µL to ICM. The samples were titrated with 0.1 N NaOH to the endpoint of the phenolphthalein indicator, a pink

were titrated with 0.1 N NaOH to the endpoint of the phenolphthalein indicator, a pink colour for the milk samples and peach for the ICM. Results were calculated as change in percent lactic acid (Δ%TA) after 8 h.

### 3.2.4 Culture media

Bacterial cultures were subcultured in M17 broth (Terzaghi and Sandine, 1975) and two modified M17 broths, one containing an increased (5x) amount of magnesium sulphate ($MgSO_4$) and one without $MgSO_4$. Steamed 10% RSM (100°C for 30 min) was also used. Single colonies were isolated and purified on M17 agar containing calcium chloride (Terzaghi and Sandine, 1975). Bulk culture media included 10% and 24% RSM, ultrafiltered milk retentate R3:1 and ICM (prepared as described in section 2.2.1). Bacterial numbers were monitored on APT (Difco) agar plates.

### 3.2.5 Bacteriophage induction of starter strains

Starter strains were treated to induce phage using slight modifications of the methods described by Huggins and Sandine (1977). Filter strips of frozen starter strains were aseptically transferred to 10 mL of M17 broth and incubated at 30°C for 16 h. A 0.1 mL aliquot of a 16 h subculture was transferred to 10 mL of fresh M17 broth, incubated at 30°C for 4 h, centrifuged at 7000 x g for 10 min, resuspended in 5 mL of sterile 0.1 M $MgSO_4$ (Fisher), transferred to a sterile pyrex petri dish (10 x 90 mm) and irradiated with UV light (Blak-Ray Lamp Model UVL-21, Ultra-Violet Products, Inc., San Gabriel, CA) at a 40 cm distance with constantly swirling the petri dish. Strains 108 and 205 were irradiated for 6, 12, 30 and 60 sec to determine the optimum exposure time. Strains 103, 202, 208, 777, 804 and 888 were irradiated for 60 sec. The irradiated cell suspensions were transferred to tubes containing 5 mL

colorimeter (Bausch and Lomb, Rochester, N. Y). The strains were also induced with mitomycin C (5 µg/mL; Sigma Chemical Company, St. Louis, MO), added to a 4 h culture grown in M17 broth. Incubation was continued at 30°C and turbidity was monitored as described above. The 24 h cultures (induced and control) were centrifuged at 7000 x g for 10 min and the supernatants were filter sterilized through 0.22 µm Millipore membranes (Millipore Corporation, Bedford, Mass).

### 3.2.6 Bacteriophage stocks

Phage stocks were prepared by adding 0.5 mL of a $10^{-4}$ dilution of bacteriophage strain to 0.5 mL of a 16 h M17 culture grown in M17 broth and 0.25 mL sterile 1.0 M $CaCl_2$ (Sigma). After 10 min of incubation at room temperature, the mixture was added to 50 mL of M17 broth and incubated overnight at 30°C. The culture was centrifuged at 7000 x g for 10 min and the supernatant was filtered through a 0.22 µm Millipore membrane. The phage stocks were stored at 2°C.

### 3.2.7 Bacteriophage assays

M17 agar plates were seeded with bacteria by adding 100 µL of an overnight M17 broth culture with 50 µL sterile 1.0 M $CaCl_2$ (1.0 M) to 5.0 mL of M17 soft (0.75%) agar (Terzaghi and Sandine, 1975), mixed at low speed on a Vortex mixer before it was poured onto the surface of M17 agar plates. The seeded plates were spotted with 10 to 20 µL of phage stock or supernatants of the induced cultures. All bacterial strains were tested with each supernatant or phage stock to determine the phage host range. The plates were dried and incubated at 30°C for 24 h under anaerobic conditions, and examined for zones of lysis or plaques. Phage titres were determined by spotting 10 µL of ten-fold dilutions of phage stock onto a lawn of a susceptible host and counting the plaques.

### 3.2.8 Bacteriophage susceptibility

Five isolated colonies of strains 205, 777, 804 and 888 were purified on M17 agar, grown in M17 broth for 16 h at 30°C and stored for 8 h at 2°C, were subcultured daily for four days. After each subculture, each strain was tested for susceptibility to phages 202 and 804. Ten colonies of strain 205 were each suspended in 1.0 mL of sterile 0.1% peptone water (Difco), and inoculated (0.1 mL) into 10 mL of M17 broth, M17 without $MgSO_4$ (M17-Mg), M17 with 5x $MgSO_4$ (M17+5xMg) and 10% RSM, incubated at 30°C for 16 h and stored at 2°C for 8 h. Each culture was subcultured 20 times in each medium at 24 h intervals and was tested for susceptibility to phage 202 for the first 11 days and on the 15th and 20th day by determining the phage titres. Colonies that were subcultured in M17-Mg were tested for phage susceptibility on standard M17 agar and overlay, as well as on M17 agar and overlay prepared without $MgSO_4$.

### 3.2.9 Bacteriophage susceptibility in bulk culture media

The bulk culture media, 10% and 24% RSM, R3:1 and ICM, were tested for their ability to inhibit phage multiplication. A 0.5 mL aliquot of an active 16 h culture of *L. cremoris* strains 205 and 777, grown in 10% RSM, was used to inoculate 50 mL of two sets of bulk culture media, one set containing approximately $1.5 \times 10^3$ plaque forming units (PFU) per mL of phage 202, the other set was used as the control. The inoculated bulk culture media were incubated at 30°C for 16 h. The Δ%TA was determined after 8 h and cell numbers, phage titres and pH (Fisher Thin Electrode with a Fisher Accumet Model 230 pH meter) were determined after 16 h of incubation. The phage titre was determined by spotting 10 µL of ten-fold dilutions of the substrate, diluted in 0.1% peptone water onto the plates, seeded with 0.1 mL of an active 10% RSM culture of the test strain. A 0.5 mL aliquot of the 16 h bulk culture was subsequently inoculated into 50 mL of 10% RSM and the Δ%TA was measured after 8 h.

All measurements were carried out in duplicate, trials to determine phage susceptibility in bulk culture media and subsequent activity in 10% RSM were done in triplicate.

## 3.3 Results

### 3.3.1 Storage stability of starter cultures at 2°C

Little or no starter activity remained when the strains were grown in 10% RSM at 30°C for 16 h and stored at 2°C for 5 days (Table 3.1). *L. cremoris* strains 804 and 888 did not grow well in 10% RSM and were therefore not used for the storage study. Starter activity was not impaired when cultures were stored in 24% RSM, R3:1 or ICM. With the exception of strain 208, starter activity of the strains stored in 24% RSM or R3:1 was approximately equal to those stored in ICM. Starter activity before storage was generally slightly lower when grown in 10% RSM compared with 24% RSM and R3:1. Cell numbers for strain 205 decreased by approximately 1 log cycle or more when stored in the bulk culture substrates (Table 3.2). Cell numbers for strain 777 stored in 10% RSM also decreased by almost 1 log cycle. Cell numbers for other strains remained the same during the 5-day storage period. The pH of the substrates ranged from 4.2 to 4.4 in 10% RSM, from 4.5 to 4.7 in 24% RSM, from 4.4 to 4.6 in R3:1 and from 5.0 to 5.1 in ICM.

### 3.3.2 Prophage induction

*L. cremoris* strains 108 and 205 were screened for optimum exposure time to UV light for prophage induction. A marked decrease in growth rate was observed when strain 108 was exposed to UV light for 30 sec or more (Fig 3.1). This was not due to lysis by phage because no plaques or zones of lysis were observed when the supernatant was spotted onto a lawn of the parent strain. Strain 205 was not affected by exposure

Table 3.1 Activity* (Δ%TA) of six strains of *L. cremoris* during storage at 2°C for 5 days in different bulk culture media.

| *L. cremoris* strain | Storage substrate and time of storage (d) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 10%RSM | | 24% RSM | | R3:1 | | ICM | |
| | 0 | 5 | 0 | 5 | 0 | 5 | 0 | 5 |
| 103 | 0.39 | 0.06 | 0.41 | 0.42 | 0.44 | 0.46 | 0.45 | 0.45 |
| 108 | 0.43 | 0.06 | 0.47 | 0.49 | 0.48 | 0.49 | 0.47 | 0.45 |
| 202 | 0.42 | 0.07 | 0.49 | 0.47 | 0.50 | 0.47 | 0.51 | 0.47 |
| 205 | 0.46 | 0.00 | 0.49 | 0.49 | 0.50 | 0.48 | 0.51 | 0.48 |
| 208 | 0.28 | 0.01 | 0.34 | 0.37 | 0.39 | 0.42 | 0.50 | 0.47 |
| 777 | 0.46 | 0.00 | 0.48 | 0.45 | 0.49 | 0.47 | 0.45 | 0.49 |

*Activity measured by incubation at 30°C for 8 h in 10% RSM, Mean of three replicate studies

Table 3.2 Viability* ($log_{10}$ of CFU/mL) of 6 strains of *L. cremoris* during storage at 2°C for 5 days in different bulk culture media.

| *L. cremoris* strain | Storage substrate and time of storage (d) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 10% RSM | | 24% RSM | | R3:1 | | ICM | |
| | 0 | 5 | 0 | 5 | 0 | 5 | 0 | 5 |
| 103 | 8.68 | 8.74 | 9.25 | 9.24 | 9.17 | 9.26 | 9.24 | 9.10 |
| 108 | 8.65 | 8.40 | 9.09 | 9.01 | 8.99 | 9.01 | 8.22 | 8.39 |
| 202 | 8.53 | 8.46 | 9.23 | 9.09 | 8.95 | 9.10 | 8.45 | 8.49 |
| 205 | 8.84 | 7.24 | 9.79 | 7.85 | 9.59 | 8.16 | 8.79 | 7.91 |
| 208 | 8.64 | 8.57 | 9.42 | 8.85 | 8.91 | 9.23 | 9.12 | 9.27 |
| 777 | 8.39 | 7.49 | 8.43 | 8.36 | 8.70 | 8.42 | 8.80 | 8.39 |

*Geometric $log_{10}$ mean count based on three studies

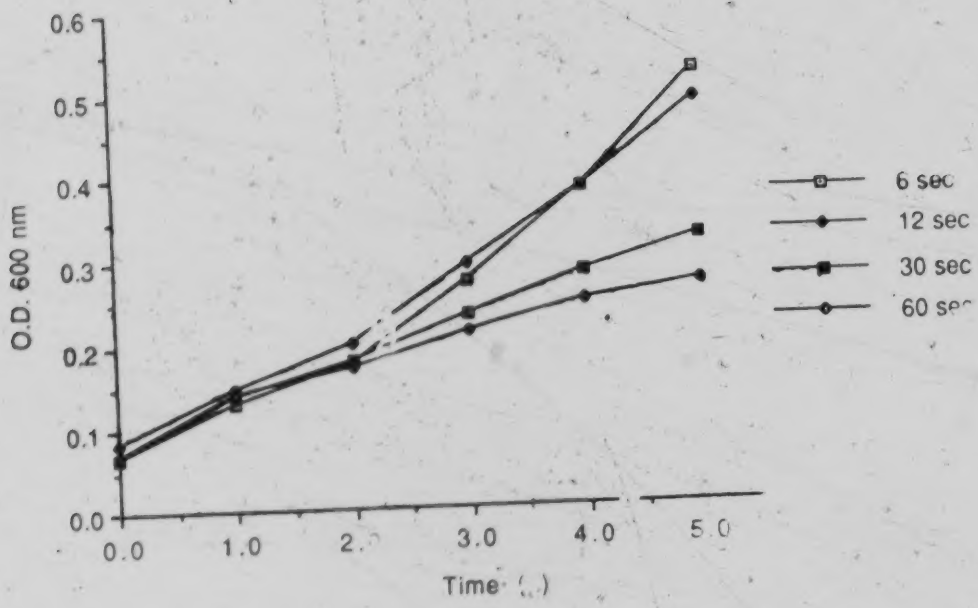


Fig 3.1 Growth of *L. cremoris* strain 108 after exposure to UV light

to UV light for 60 sec. None of the strains tested liberated prophage when induced with UV light for 60 sec or with mitomycin C (5 µg/mL), even though all strains were sensitive to mitomycin C.

### 3.3.3 Susceptiblity of strains to phage 202 and 804

Variable results were obtained when the host ranges of phages 202 and 804 were determined. Phage sensitivity of some strains varied from one trial to the next. This led to the monitoring of phage susceptibility as a function of repeated subculture. Five colonies of phage sensitive strains 205, 777, 804 and 888 were isolated and examined for phage susceptibility at each subculture. All four of these strains were sensitive to phage 202 and strains 804 and 888 were also sensitive to phage 804. After four subcultures in M17 broth all bacterial strains lost their sensitivity to the phage strains. Loss of phage sensitivity was progressive, increasing with number of subcultures.

The effect of broth medium and milk (RSM) as well as concentration of magnesium sulphate in the broth medium, on the loss of phage sensitivity as a result of repeated subculture was examined. For this study, 10 colonies of strain 205 were isolated and subcultured in M17 broth, M17-Mg, M17+5xMg and 10% RSM. The presence of $MgSO_4$ in M17 broth resulted in the loss of phage susceptibility. After 20 subcultures, none of the colonies subcultured in M17-Mg had lost their susceptibility to phage 202, regardless of whether the bacterial strain was plated on M17 agar with or without $MgSO_4$. After 6 subcultures in M17, 8 of the 10 cultures had become insensitive to the phage; after 20 subcultures the two remaining cultures were still sensitive to phage 202 and their phage titres were unchanged at approximately 5 x $10^8$ PFU/mL. After 6 subcultures in M17+5xMg, one culture had lost its phage

sensitivity and two failed to grow; after 10 subcultures, the 7 remaining cultures had also lost their phage susceptibility. Phage sensitivity was not regained after 15 subcultures for cultures grown in either M17 or M17+5xMg. After 20 subcultures in 10% RSM, all 10 cultures retained their sensitivity to phage 202.

### 3.3.4 Bacteriophage activity in bulk culture media

The four bulk culture media were evaluated for their effect on phage susceptibility of *L. cremoris* strains 205 and 777 to phage 202. The bulk culture media were inoculated with approximately 1.5 x $10^3$ PFU of phage per mL. After incubation for 16 h phage titres reached levels of $10^9$ PFU/mL or higher in milk substrates compared with $10^4$ PFU/mL or lower in ICM (Table 3.3). Decreased starter activity was observed in bulk culture media containing phage (Table 3.4), except in the phage inhibitory medium ICM even though the difference in viable cell numbers in the phage containing substrates was less than 1 log cycle than in the control substrates after 16 h of incubation. When bulk cultures propagated in the presence of phage were subcultured into 10% RSM, the cultures that were grown in milk based media had little or no activity (Table 3.5). When strain 777 was used as host for phage 202 in ICM no phage particles could be detected after 16 h of incubation in the substrate and no loss of activity was observed when the cultures were subsequently inoculated into 10% RSM (Table 3.5). However, a large loss of activity was observed for strain 205.

Table 3.3 Phage titre* ($log_{10}$ PFU/mL) of phage 202 when grown on *L. cremoris* strains 205 and 777 in different bulk culture media at 30°C for 16 h.

| Substrate | strain 205 | strain 777 |
|---|---|---|
| 10% RSM | 9.62 | 9.31 |
| 24% RSM | 10.72 | 9.71 |
| R3:1 | 10.95 | 10.11 |
| ICM | 4.26 | <3.00 |

*Geometric $log_{10}$ mean count results from three studies

Table 3.4 Activity* (Δ%TA) of *L. cremoris* strains 205 and 777 after inoculation in different bulk culture media in the presence and absence of phage 202.

| | strain 205 | | strain 777 | |
|---|---|---|---|---|
| Substrate | Control | Phage | Control | Phage |
| 10% RSM | 0.47 | 0.20 | 0.45 | 0.28 |
| 24% RSM | 0.44 | 0.38 | 0.31 | 0.22 |
| R3:1 | 0.59 | 0.22 | 0.40 | 0.25 |
| ICM | 0.60 | 0.59 | 0.74 | 0.67 |

*Activity measured by incubation at 30°C for 8 h in different bulk culture media. Mean of three replicate studies.

Table 3.5 Activity* (Δ%TA) of *L. cremoris* strains 205 and 777 after growth in different bulk culture media in the presence and absence of phage 202.

| | strain 205 | | strain 777 | |
|---|---|---|---|---|
| Substrate | Control | Phage | Control | Phage |
| 10% RSM | 0.46 | 0.00 | 0.46 | 0.00 |
| 24% RSM | 0.49 | 0.01 | 0.48 | 0.01 |
| R3:1 | 0.50 | 0.07 | 0.49 | 0.02 |
| ICM | 0.51 | 0.20 | 0.45 | 0.46 |

*Activity measured by incubation at 30°C for 8 h in 10% RSM, Mean of three replicate studies.

### 3.4 Discussion

Because lysogenic starter strains have often been implicated as a major source of phage (Huggins and Sandine, 1977; Heap *et al.*, 1978; Terzaghi and Sandine, 1981), the strains used in this study were studied for lysogeny, using both UV light and mitomycin C as inducing agents. The slower growth rates observed when strain 108 was treated with UV light for 30 sec or more indicate that phage lysis may have occurred. However, no plaques or zones of lysis were observed when the supernatant was spotted onto the parent strain. The cells may have been damaged by UV light, because UV light is known to cause DNA damage that must be repaired before cell division can take place (Ingraham *et al.*, 1983). Slower growth rates observed in the presence of mitomycin C may also indicate phage lysis, however, no plaques or zones of lysis were observed on the parent or other strains. Mitomycin C is a mutagenic agent that binds covalently to DNA and prevents the separation of the two strands because of the bridges that are formed between the two strands of DNA (Lancini and Parenti, 1982). The sensitivity to mitomycin C in all of the strains tested may result from damage to the cell. The strains tested (in duplicate) in this study did not liberate any prophage.

Cultures grown in M17 broth lose their phage sensitivity after repeated subculture, indicating that M17 may not be an ideal medium for phage assays. There are several possible explanations for the loss of phage sensitivity. The bacterial cell may mutate and become insensitive to the phage. However, all of the cultures used in this study originated from a single colony and the results obtained were consistent. Phage resistance mechanisms have been linked to plasmids (Sanders, 1988). Phage resistant cells would not be expected to outgrow phage sensitive cells, because plasmids increase cell doubling times thus slowing cell growth. The most plausible explanation is that when the bacterial strains are exposed to the phage they enter the lysogenic state as opposed to the lytic cycle. Turbid plaques, as were observed as an intermediate

step in loss of phage sensitivity, are indicative of lysogeny (Terzaghi and Sandine, 1975). Repeated subculturing may prompt the cells to enter the lysogenic state to the point where all of the cells have become lysogenic and no plaques or zones of lysis become visible. This theory could be confirmed by isolating colonies from inside a turbid plaque, inducing them with either UV light or mitomycin C to release the prophage and testing the supernatant on a sensitive host as well as examining the supernatant for phage particles under the electron microscope. Because the focus of this study centered around phage susceptibility in bulk culture media, further studies were not carried out.

Cells remained sensitive to phage in the absence of $MgSO_4$. After infection, cell walls are stabilized by $Mg^{2+}$ (Mathews, 1971). In the absence of $Mg^{2+}$, cations may leak from the cell and cell lysis may be encouraged. Cells subcultured in M17 and M17+5xMg are protected from cell leakage and the phage enters the lysogenic state. Cells subcultured in 10% RSM maintain their phage sensitivity. Therefore, it may be desirable to culture bacterial strains in M17-Mg or 10% RSM to determine host ranges of phages.

ICM is an effective phage inhibitory medium whereas the milk based bulk culture media encourage phage multiplication. Phage titres in 24% RSM and R3:1 were higher than those observed in 10% RSM, possible because higher cell numbers can be achieved in 24% RSM and R3:1. Cell numbers were very high even in the presence of phage. These cells may either be resistant to the phage or carry the phage in a lysogenic state. When the three milk-based bulk cultures were inoculated into 10% RSM, virtually all cells were considered to be lysed because little or no activity was detected after incubation for 8 h. ICM is effective in controlling phage. Phage titres with strain 205 increased approximately ten-fold in ICM. When cultures grown in ICM were used to inoculate 10% RSM, a loss in activity was observed similar to that observed in 10% RSM as a bulk culture medium. Because the phage particles

proliferated in ICM when strain 205 was used as a host but decreased in the presence of strain 777, the ICM medium cannot be considered responsible for the loss of phage but rather the bacterial strain 777. It is possible that *L. cremoris* strain 777 may contain restrictive capabilities that are activated when grown in ICM.

The activity of starter strains stored at 2°C for 5 days in 24% RSM and R3:1 is comparable to that of cultures stored in ICM, confirming results obtained in chapter 2. But, even though 24% RSM and UF milk retentates are suitable media for producing active bulk cultures, they do not possess the phage inhibitory properties that are characteristic of ICM and therefore, in order to avoid economic losses, the use of ICM would be preferred over increased solids RSM and UF retentates for the manufacture of bulk cultures. Phage susceptibility could be decreased by the addition of phosphates to 24% RSM and UF milk retentates, which would tie up the available calcium as well as increase the buffering capacity of the medium and as a result, produce highly active cultures.

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# 4. EFFECT OF STORAGE CONDITIONS ON ACTIVITY AND VIABILITY OF STARTER CULTURES

## 4.1 Introduction

Starter cultures lose their activity and suffer damage upon prolonged storage below pH 5.0 in milk due to the exposure to high hydrogen ion concentrations (Lawrence *et al.*, 1976). This causes an increased lag phase on subculture that is attributed to a reduction in the specific activities of cytoplasmic enzymes (Harvey, 1965). Storage of starter cultures at low pH not only results in cellular injury but also promotes loss of plasmids that can result in an increased proportion of slow acid producing cells in the culture (Sandine, 1985). Fully grown cultures of *L. cremoris* in milk stored for 5 days in 10% RSM at refrigeration temperatures displayed little or no activity on subculture after 8 h of incubation at 30°C (see sections 2.3.2; 3.3.1). Although freezing and storage of starter cultures in liquid nitrogen is an effective means of preserving cell viability and activity (Peebles *et al.*, 1969; Lloyd, 1975), frozen storage at -196°C is very expensive and only small quantities can be frozen at any one time. Most cultures can be stored for short times at -20 to -40°C, but death, metabolic injury and loss of proteinase activity were observed when *L. lactis* was frozen and stored at -20°C (Cowman and Speck, 1963; Moss and Speck, 1963). Variable results were reported for storage at -40°C and above (Gibson *et al.*, 1965, 1966; Stadhouders *et al.*, 1971; Lloyd, 1975). Neutralization of cultures prior to freezing minimizes loss of cell viability and activity (Lamprech and Foster, 1963; Gilliland and Speck, 1974).

Cryoprotectants such as glycerol and lactose have been added to cultures in attempts to improve their survival during freezing and frozen storage. Freezing and storage of lactococci at -37°C in the presence of 7.5% lactose allowed storage for up to 3 months without loss of activity (Stadhouders *et al.*, 1971) but this has been reported

to be ineffective by others (Efstathiou *et al.*, 1975). Cryoprotectants protect cultures from freeze injury at pH 5.0 but they give no additional benefit at neutral pH (Stadhouders *et al.*, 1971). Storage temperature, however, is the single most important factor in maintaining starter activity (Bauman and Reinbold, 1966). The optimum storage temperatures for starter cultures without cryoprotectants or neutralization are -40°C and below.

The objectives of this study were to screen for the viability and activity of *L. cremoris* strains 108 and 205 after freezing and storage at -70°C when grown in 10% RSM, 24% RSM and UF retentate R3:1 and to examine if added protection from freeze injury was achieved by neutralizing the cultures prior to freezing. Also, to determine the critical pH for loss of activity and the effect of hydrogen ions and lactate ions on the loss of activity by strain 205 at 2°C.

## 4.2 Materials and Methods

### 4.2.1 Bacterial strains

*Lactococcus lactis* ssp. *cremoris* (*L. cremoris*) strains 108 and 205 were retrieved from frozen storage (section 2.2.4), propagated in M17 broth (Terzaghi and Sandine, 1975) and subcultured twice in 10% RSM (section 2.2.1) before use in experiments. The inoculum size was standardized at 1% unless otherwise mentioned. Bacterial numbers were determined on APT (Difco Laboratories Inc., Detroit, MI) agar plates.

### 4.2.2 Preparation of cultures for freezing

An active 10% RSM culture was inoculated into two 50 mL aliquots of 10% RSM, 24% RSM and UF retentate R3:1 (section 2.2.1) and incubated at 30°C for 8 h. One set of cultures was neutralized to pH 6.5 with 33% NaOH. The cultures were

dispensed in 2.5 mL quantities in sterile vials and frozen at -70°C. The samples were thawed in a 30°C waterbath for 5 min.

### 4.2.3 Starter activity

The activity of *L. cremoris* strains 108 and 205 were determined by measuring the change in total titratable acidity (Δ%TA) in 10% RSM at 30°C (section 3.2.8).

### 4.2.4 Turbidity measurement of starter cultures

The turbidity of the starter cultures in milk was determined by the method described by Thomas and Turner (1977). The inoculated milk was diluted 10-fold with 0.2% EDTA adjusted to pH 12.0, turbidity was determined spectrophotometrically at 480 nm (Spectronic 20, Bausch and Lomb, Rochester, N. Y).

### 4.2.5 Measurement of proteolytic activity

The proteinase activity of *L. cremoris* strains 108 and 205 was determined before and after 5 days of storage at 2°C and before and after 1, 14 and 56 days of storage at -70°C. An active 10% RSM culture (5 mL) was used to inoculate 50 mL of 10% RSM containing 0.25 mL of toluene to prevent cell growth (Peebles *et al.*, 1969). The inoculated milk was incubated at 30°C. At 4 h intervals, a 5 mL sample was removed to which 1 mL of distilled water was added and 10 mL of 0.75 N trichloroacetic acid (TCA) (BDH Inc., Edmonton, AB) was added while mixing on a vortex shaker at a low speed. After incubation at room temperature for 10 min, the solution was filtered through a Whatman #2 filter paper and stored at -70°C for assay by an o-phthaldialdehyde (OPA) spectrophotometric assay (Church *et al.*, 1983). The OPA reagent was prepared by combining 50 mL of 100 mM sodium tetraborate (BDH), 5 mL of 20% (w/w) sodium dodecyl sulphate (BDH), 80 mg of OPA (BDH) dissolved in 2 mL of methanol and 200 µl of ß-mercapto-ethanol (BDH) in a 100 mL volumetric

flask made to volume with distilled water. The flask was wrapped in aluminum foil and held at room temperature for 2 h to allow for colour development. This reagent was made fresh daily. A 150 µl volume of the TCA filtrate was added to 3 mL of OPA reagent, incubated for 2 min and the absorbance was measured at 340 nm with a spectrophotometer (Spectronic 20). Samples were analyzed in duplicate.

### 4.2.6 Treatment of cultures for determination of acid sensitivity

Active 10% RSM cultures were inoculated into 5 bottles containing 100 mL of 10% RSM and were incubated at 30°C for 16 h. Bacterial cells were harvested by neutralizing the fermented milk to pH 6.5 with 33% NaOH and adding sodium citrate to a final concentration of 1% (Sandine and Elliker, 1970). This mixture was incubated at room temperature for 30 min to solubilize the milk proteins and centrifuged at 8000 x g for 10 min at 4°C. The bacterial pellet was resuspended in fresh 10% RSM and 10% RSM adjusted to pH 4.2 with either HCl or lactic acid. One fermented milk sample was adjusted to pH 4.7 with NaOH and one was left untreated. These cultures were stored at 2°C for 5 days and their activity and cell numbers were determined before and after storage.

An active 10% RSM culture was used to inoculate four 100 mL batches of 10% RSM which were incubated at 30°C for 16 h. An untreated fermented milk sample and three batches adjusted to pH 4.3, 4.5 and 4.7 with NaOH, were stored at 2°C for 5 days. The cell numbers and activity were determined before and after storage in 10% RSM. The activity of the untreated fermented culture (pH 4.1) was measured in 10% RSM as well as in 10% RSM containing 0.5% casamino acids (Difco). After 5 days of storage, activity of all cultures was measured after 8 and 12 h, and after 24 h for the culture stored at pH 4.1. The activity of a subculture of the starter stored at pH 4.1 and grown for 24 h in 10% RSM and 10% RSM with 0.5% casamino acids was determined after 8 h. Cells were screened for proteolytic activity by plating on Fast Slow Differentiating

Agar (FSDA)(Sandine, 1985) and for their ability to metabolize lactose on lactose indicator agar. Trials were done in triplicate.

### 4.3 Results

Ability to metabolize lactose was not impaired when *L. cremoris* strains 108 and 205, grown in 10% RSM, 24% RSM and R3:1, were frozen and stored at -70°C (Tables 4.1 and 4.2). The activity of neutralized 24% RSM and R3:1 cultures of *L. cremoris* strain 108 was lower than that of unadjusted cultures (Table 4.1), but this difference was decreased after freezing. The neutralized 10% RSM culture had a greater activity than the unadjusted culture, even after 56 days of frozen storage. Higher activities were observed when strain 108 was grown and stored in 24% RSM and R3:1 compared with 10% RSM. For strain 205, the opposite was observed.

There was no change in cell viability after 56 days of frozen storage at -70°C. Cultures of strain 108 (Table 4.3) grown in R3:1 reached higher cell numbers than those grown in 10% RSM and 24% RSM, but numbers decreased slowly over the 56 day storage period. Cell numbers did not decrease for cultures grown and stored in 10% RSM and 24% RSM. Cell numbers remained stable for *L. cremoris* strain 205 (Table 4.4) and were approximately the same in all culture substrates.

Cell growth, measured by an increase in turbidity at 480 nm (Fig 4.1) in 10% RSM, was not delayed after storage at -70°C for 56 days. Results are shown for strain 108 in 10% RSM with and without neutralization. Similar results were observed for strains 108 and 205 in all three substrates, with and without neutralization. Fig 4.2 illustrates that cell growth occurs at approximately the same rate after 56 days storage at -70°C, regardless of storage substrate. Maximum cell numbers were generally reached after 8 h of incubation at 30°C.

Table 4.1 Activity* (Δ%TA) of *L. cremoris* strain 108 before and after storage at -70°C in different substrates with and without neutralization.

| | Storage time (days)* at -70°C | | | |
|---|---|---|---|---|
| Substrates | before | 1 | 14 | 56 |
| 10% RSM | 0.28 | 0.30 | 0.28 | 0.31 |
| 10% RSM N** | 0.37 | 0.37 | 0.34 | 0.38 |
| 24% RSM | 0.44 | 0.29 | 0.28 | 0.42 |
| 24% RSM N** | 0.28 | 0.31 | 0.33 | 0.43 |
| R3:1 | 0.48 | 0.39 | 0.38 | 0.43 |
| R3:1 N** | 0.36 | 0.39 | 0.35 | 0.41 |

*Activity measured by incubation at 30°C for 6 h in 10% RSM
**N=neutralized

Table 4.2 Activity* (Δ%TA) of *L. cremoris* strain 205 before and after storage at -70°C in different substrates with and without neutralization.

| Substrates | Storage time (days) at -70°C | | | |
|---|---|---|---|---|
| | before | 1 | 14 | 56 |
| 10% RSM | 0.35 | 0.28 | 0.31 | 0.28 |
| 10% RSM N** | 0.36 | 0.33 | 0.32 | 0.30 |
| 24% RSM | 0.27 | 0.21 | 0.25 | 0.25 |
| 24% RSM N** | 0.25 | 0.24 | 0.26 | 0.23 |
| R3:1 | 0.27 | 0.23 | 0.24 | 0.25 |
| R3:1 N** | 0.23 | 0.22 | 0.20 | 0.23 |

*Activity measured by incubation at 30°C for 6 h in 10% RSM
**N=neutralized

Table 4.3 Viability ($log_{10}$ CFU/mL) of *L. cremoris* strain 108 before and after storage at -70°C in different substrates with and without neutralization.

| | Storage time (days) at -70°C | | | |
|---|---|---|---|---|
| Substrates | before | 1 | 14 | 56 |
| 10% RSM | 7.78 | 7.83 | 7.76 | 8.28 |
| 10% RSM N* | 7.72 | 7.76 | 7.92 | 8.01 |
| 24% RSM | 7.74 | 7.24 | 7.99 | 7.63 |
| 24% RSM N* | 7.80 | 7.74 | 7.76 | 7.88 |
| R3:1 | 8.64 | 8.28 | 8.33 | 7.98 |
| R3:1 N* | 8.44 | 8.44 | 8.21 | 7.83 |

*N=neutralized

Table 4.4 · Log CFU/mL of *L. cremoris* strain 205 before and after storage at -70°C in different substrates with and without neutralization.

| Substrates | Storage time (days) at -70°C | | | |
|---|---|---|---|---|
| | before | 1 | 14 | 56 |
| 10% RSM | 8.99 | 8.89 | 8.89 | 8.90 |
| 10% RSM N* | 9.08 | 8.99 | 9.07 | 9.11 |
| 24% RSM | 9.15 | 9.01 | 9.01 | 9.11 |
| 24% RSM N* | 9.20 | 9.26 | 9.07 | 9.20 |
| R3:1 | 9.00 | 9.00 | 8.92 | 9.11 |
| R3:1 N* | 9.02 | 9.11 | 9.01 | 9.11 |

*N=neutralized

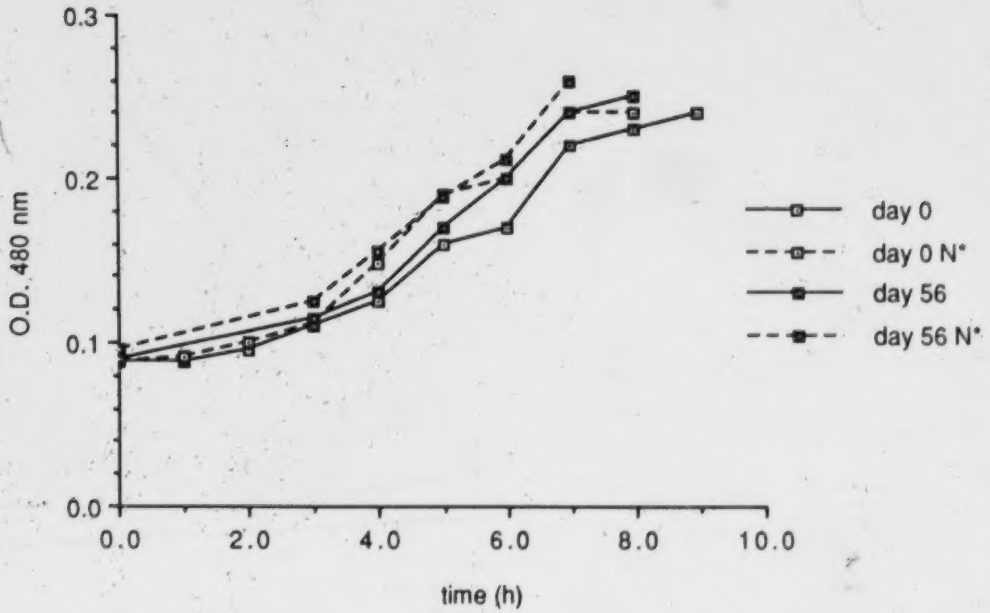


Fig 4.1 Growth (O. D. 480) of *L. cremoris* strain 108 at 30°C before and after 56 days of storage at -70°C with and without neutralization.

*N=neutralized

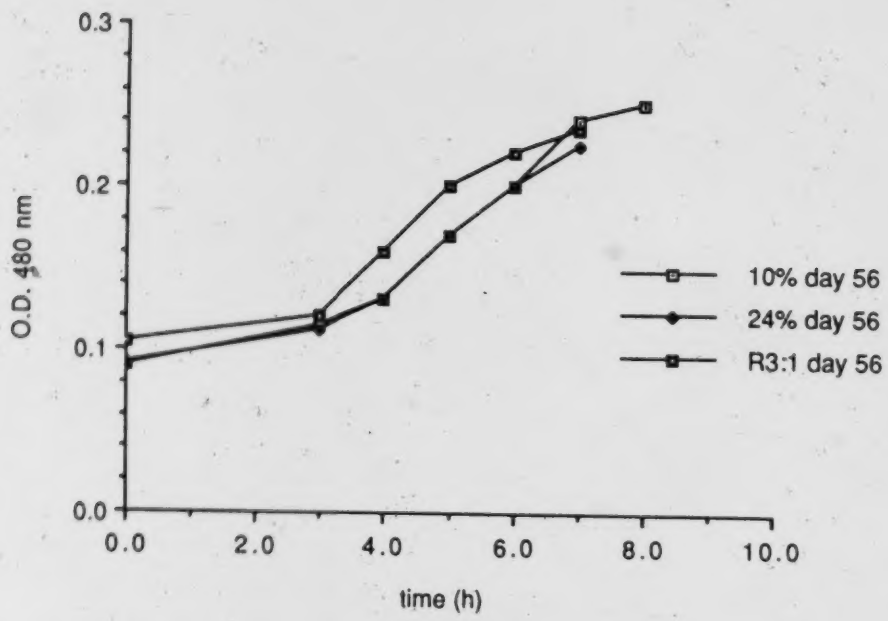


Fig 4.2 Growth (O. D. 480) of *L. cremoris* strain 205 at 30°C after 56 days of storage at -70°C in different substrates

The proteolytic activity of strain 108 was impaired after 14 days of frozen storage at -70°C in 10% RSM (Fig 4.3). The neutralized culture showed a loss of proteolytic activity, but the effect was less dramatic than that observed for the unadjusted culture. The proteolytic activity of strain 205 decreased between 14 and 56 days of storage (Fig. 4.4) for cultures stored in both the unadjusted and neutralized substrates. The proteolytic activity did not decrease when the strains were grown and stored in 24% RSM. A decrease in proteolytic activity occurred with increased storage time for neutralized and unadjusted cultures of strain 108 in R3:1. Variable activity was observed for strain 205 in R3:1 (data not shown). The proteolytic activity of the cultures in neutralized storage media was higher than that of the unadjusted cultures for both test strains. After 56 days of storage at -70°C, the proteolytic activity of strain 108 was lower in 10% RSM cultures but little difference was observed between the cultures stored in neutralized media (Fig 4.5). In contrast, the proteolytic activity of strain 205 was higher than that observed for 24% RSM and R3:1 cultures (Fig 4.6).

Starter cultures lost activity during prolonged storage at low pH in milk. Cells were harvested from fermented milk and resuspended in 10% RSM adjusted to pH 4.2 with either lactic acid or HCl. The results shown in Table 4.5 indicate that the loss of activity is primarily due to the effect of the lactate ion. When cells were stored at 2°C for 5 days in 10% RSM adjusted to pH 4.2 with HCl, cell numbers and activity the same; however, when the 10% RSM was adjusted to pH 4.2 with lactic acid, no colonies were detectable after plating and no activity was observed when subcultured into 10% RSM after 5 days of storage.

The cells resuspended in fresh 10% RSM without further treatment actively metabolized lactose during the 5 day storage period, but their numbers remained constant, indicating that lactose metabolism was uncoupled from growth (Table 4.5). No loss of activity occurred and cell numbers remained constant when the pH was

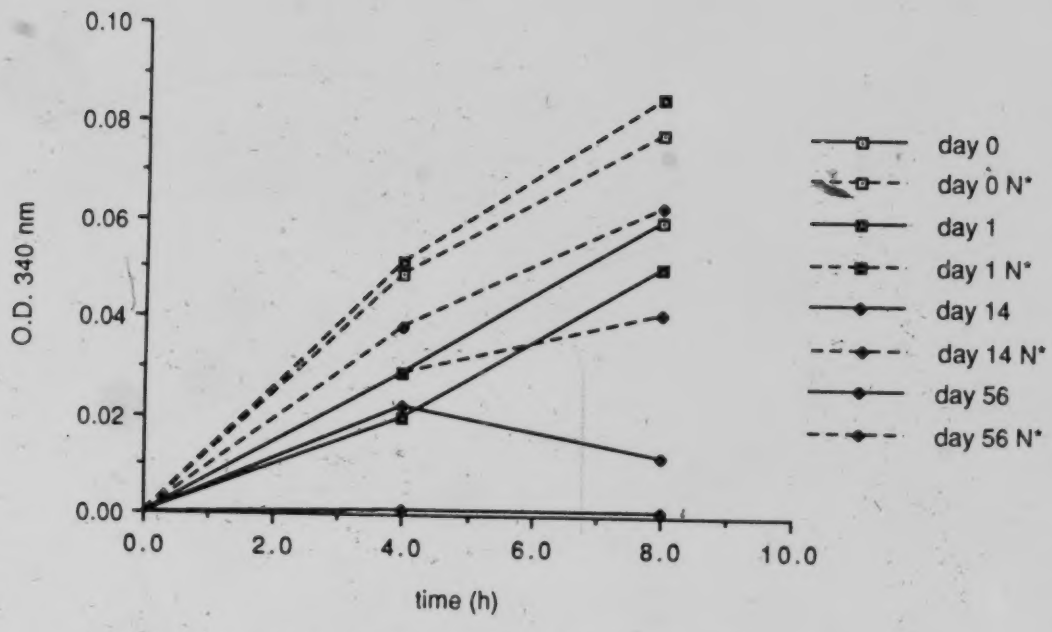


Fig 4.3 Effect of time of storage at -70°C in 10% RSM on proteolytic activity of *L. cremoris* strain 108 in 10% RSM at 30°C.

*N=neutralized

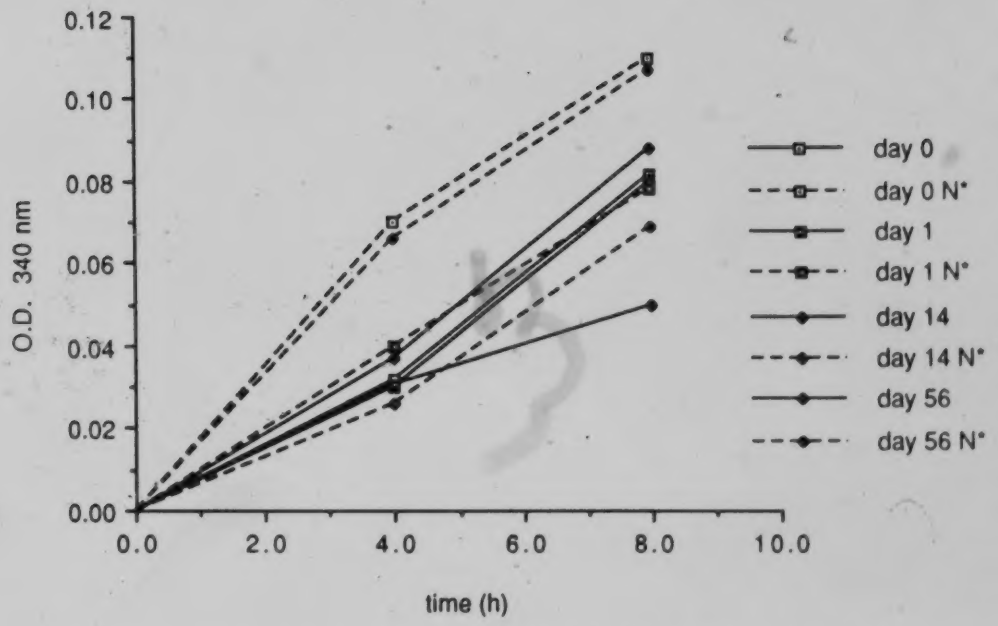


Fig 4.4 Effect of time of storage at -70°C in 10% RSM on proteolytic activity of *L. cremoris* strain 205 in 10% RSM at 30°C.

*N=neutralized

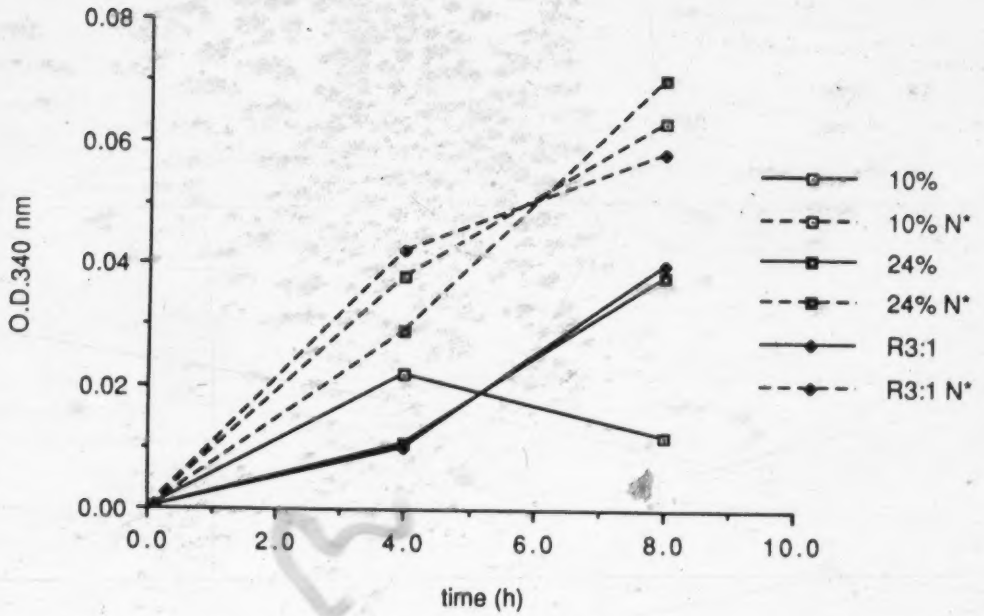


Fig 4.5 Effect of storage medium on proteolytic activity of *L. cremoris* strain 108 in 10% RSM at 30°C after storage at -70°C for 56 days.

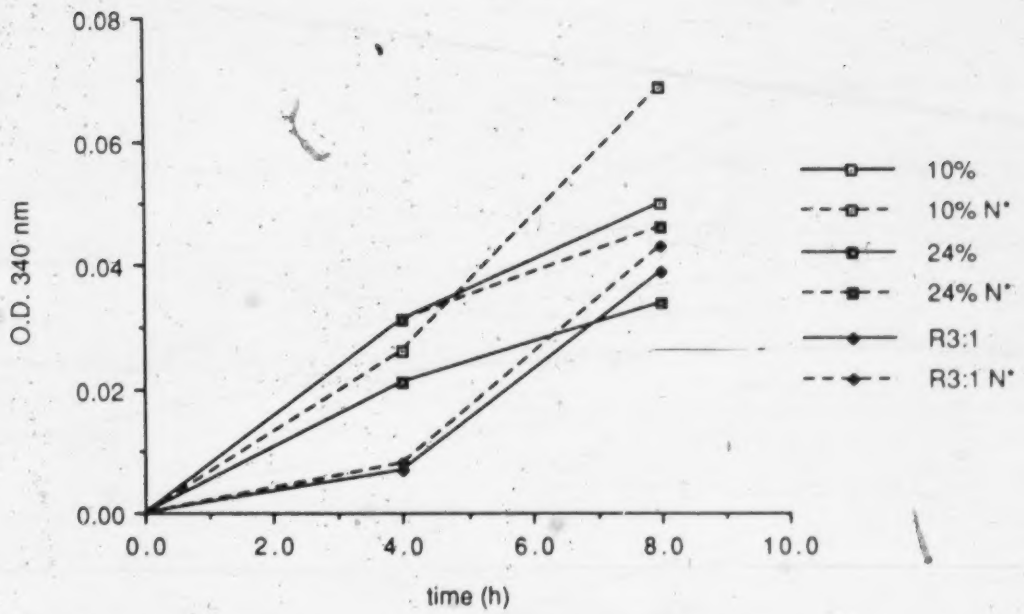


Fig 4.6 Effect of storage medium on proteolytic activity of *L. cremoris* strain 205 in 10% RSM at 30°C after storage at -70°C for 56 days.

Table 4.5 Effect of lactic acid and HCl on the activity and viability of *L. cremoris* strain 205 stored at 2°C for 5 days*.

| | Δ%TA in 10% RSM after 8 h | | Δ%TA in substrate after 16 h | | log cfu/ml in substrate after 16 h | |
|---|---|---|---|---|---|---|
| Substrate | day 0 | day 5 | day 0 | day 5 | day 0 | day 5 |
| RSM pH 4.2 | 0.43 | 0.004 | 0.489 | 0.577 | 8.92 | 8.11 |
| RSM pH 4.7 | 0.42 | 0.42 | 0.441 | 0.541 | 8.92 | 8.90 |
| fresh RSM | 0.38 | 0.34 | 0.087 | 0.489 | 8.72 | 8.79 |
| RSM pH 4.2 (L.A.)** | 0.45 | 0.00 | 1.190 | 1.227 | 8.78 | <3.70 |
| RSM pH 4.2 (HCl) | 0.37 | 0.30 | 0.610 | 0.635 | 8.72 | 8.59 |

*Mean of three replicate studies
**L.A.=adjusted with lactic acid

raised to 4.7. This led to the determination of the critical storage pH for *L. cremoris* strain 205 (Table 4.6). A decrease in cell numbers was observed only when cultures were stored at pH 4.1. At higher pH levels, cell numbers remained constant (Table 4.6). The critical pH for storage falls around pH 4.5 (Table 4.7). A 9% loss of acitivity was observed when cultures were stored at pH 4.5 but a 36% loss of activity was observed when cultures were stored at pH 4.3. The activity was regained after an additional 4 h of incubation. The culture stored at pH 4.1 was still in the lag phase after incubation in 10% RSM at 30°C for 12 h, but it regained its full activity within 24 h. The activity of a 24 h subculture after incubation in 10% RSM for 8 h was comparable to the activity before storage. When the activity of the culture stored at pH 4.1 was determined in 10% RSM containing 0.5% casamino acids, the results were similar to those obtained in 10% RSM.

No prt$^-$ cells were detected when cells were plated on FSDA, indicating that the cells had not lost their proteolytic activity during storage at 2°C for 5 days. These results were confirmed by the proteinase activity assay on strains 108 and 205. A decrease in activity was observed for both strains after storage for 5 days (Fig 4.7). Strain 108 had a higher proteolytic activity than strain 205. All cells were able to ferment lactose based on the fact that no lac$^-$ colonies were observed when culture dilutions were plated on lactose indicator agar.

## 4.4 Discussion

Loss of activity observed after storage was not plasmid mediated because neither lac$^-$ nor prt$^-$ phenotypes were observed. Loss of activity was attributed to cell injury because cells exposed to pH levels below 4.5 demonstrated a reduced activity, which was fully recovered after 12 and 24 h when stored at pH 4.3 and 4.1, respectively. Normal activity was regained after one subculture. The extended lag phase observed in cultures stored at pH 4.1 could be due to the combined effect of acid injury and cell

Table 4.6 Activity (Δ%TA) and viable count of *L. cremoris* strain 205 grown in 10% RSM for 16 h and stored at different pH levels at 2°C for 5 days*.

| | Substrate Δ%TA | | Substrate log CFU/mL | |
|---|---|---|---|---|
| Substrate pH | day 0 | day 5 | day 0 | day 5 |
| 4.1 | 0.505 | 0.465 | 9.04 | 7.52 |
| 4.3 | 0.395 | 0.375 | 9.08 | 8.63 |
| 4.5 | 0.335 | 0.385 | 9.08 | 8.90 |
| 4.7 | 0.295 | 0.395 | 8.90 | 8.88 |

*Mean of three replicate studies

Table 4.7 Activity* (Δ%TA) in 10% RSM by *L. cremoris* strain 205 after growth in 10% RSM and storage at different pH levels at 2°C for 5 days.

| Substrate pH | Δ%TA | | | |
|---|---|---|---|---|
| | day 0 | day 5 | | |
| | 8 h | 8 h | 12 h | 24 h |
| 4.1 | 0.43 | 0.003 | 0.03 | 0.55 |
| 4.3 | 0.44 | 0.28 | 0.52 | |
| 4.5 | 0.43 | 0.40 | 0.57 | |
| 4.7 | 0.44 | 0.43 | 0.53 | |

*Activity measured by incubation at 30°C in 10% RSM, Mean of three replicate studies.

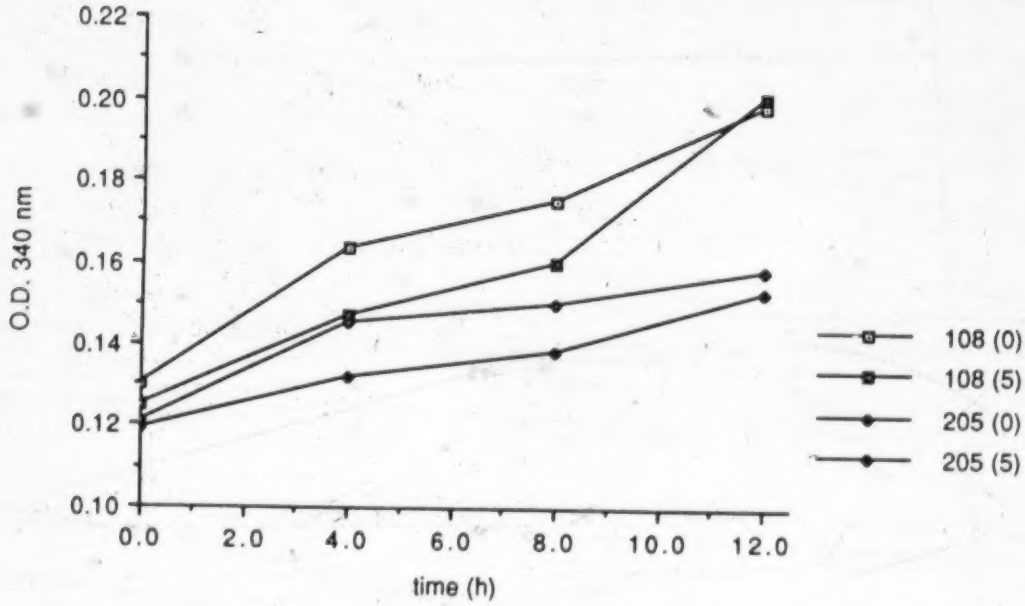


Fig 4.7 Proteinase activity of *L. cremoris* strain 108 and 205 after storage in 10% RSM at 2°C for 5 days.

death, because cell numbers were reduced 1.5 log cycles while cultures stored at pH 4.3 decreased less than 0.5 log cycle.

Reduced proteinase activity was observed after storage for both strains 108 and 205. This illustrates that the proteolytic enzymes were impaired but that their activity was not destroyed. The addition of toluene to the milk inhibits cell growth but it does not impair enzyme activity. This assay measures the activity of the enzymes associated with the cells. Synthesis of new proteolytic enzymes does not occur without cell growth (Peebles *et al.*, 1969).

The injury observed is caused primarily by the lactate ion as opposed to the hydrogen ion. Because lactic acid is a weak acid, it is present in both dissociated and undissociated forms. More lactic acid is needed to achieve the same pH than is the case with a strong acid. The pKa of lactic acid is 3.08. The undissociated form of lactic acid is responsible for the antimicrobial effect because uncharged molecules can penetrate the cell more readily than charged molecules (Ingram *et al.*, 1956). The proportion of undissociated acid is 39.2% at pH 4.0 and 6.05% at pH 5.0 (ICMSF, 1980). Exposure to low pH for prolonged periods of time may also reversibly denature internal structures and enzymes which are repaired when organisms are subcultured into a fresh medium (Marquis *et al.*, 1973; Lawrence *et al.*, 1976).

Neutralization of the cultures before freezing was of no additional benefit when cell viability, growth and lactose metabolism were evaluated. In fact, starter activity of neutralized 24% RSM and R3:1 cultures of strain 108 was lower than the unadjusted cultures before freezing. This may be due to the presence of high $Na^+$ concentrations. Peebles *et al.* (1969) suggested that reduced activity of starter cultures neutralized with NaOH may be due to inhibitory effects of the sodium ion. More NaOH is required to neutralize the 24% RSM and R3:1 cultures. However, the benefits of neutralization are realized when the proteolytic activity of the cultures is

assessed, because the proteolytic activity of the neutralized cultures was higher than in the unadjusted cultures. Indicating that the proteolytic enzymes are acid sensitive.

Conclusive observations can not be made with regards to optimum storage medium for frozen storage because of variations between strains. For strain 108, small differences were observed between all three neutralized cultures but optimum activity for strain 205 was achieved when stored in neutralized 10% RSM, little difference was observed between the neutralized 24% RSM and R3:1 cultures.

Loss of proteolytic activity did not impair cell growth or lactose metabolism. The primary function of the proteolytic system is to hydrolyze milk protein and make peptides available for growth. If these enzymes are in short supply, cell growth will be slower and less lactose will be metabolized. The cultures did not display an increased lag phase after frozen storage, which indicates that the cells were not injured during 56 days of storage. This study screened the effect of freezing over a relatively short storage period. Even at low temperature of -70°C, loss of proteolytic activity was detected when the cultures were stored under acidic conditions. Therefore, neutralization of cultures before frozen storage is recommended.

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# 5. GENERAL DISCUSSION AND CONCLUSIONS

Traditionally, cheese is made from pasteurized milk or skim milk powder. These substrates were also widely used to prepare bulk starter cultures for cheese manufacture. Because starter cultures are sensitive to the low pH of fermented milk and are prone to attack by phage in milk, pH controlled phage inhibitory media were developed (Richardson *et al.*, 1977; Sandine and Ayers, 1983). The pH of these media does not fall below 5.0 and thus prevents acid injury of the bacteria and they contain high amounts of phosphate and other chelating agents that tie up available calcium, thus inhibiting phage multiplication.

Since 1969, ultrafiltered (UF) milk retentates have been successfully used for manufacture of camembert (Maubois *et al.*, 1969), cottage cheese (Mattews *et al.*, 1976; Kosikowski, 1979; Kealy and Kosikowski, 1981; Athar *et al.*, 1983; Kosikowski *et al.*, 1985a), cream cheese (Covacevich and Kosikowski, 1977) and Cheddar cheese (Chapman *et al.*, 1974; Kosikowski, 1979; Green *et al.*, 1981; Kosikowski *et al.*, 1985b). One of the problems encountered in cheese manufactured from UF retentates is the high amount of lactic acid that starter cultures are required to produce to overcome the high buffering capacity of the UF retentates in order to reach the desired pH for cheese making. However, this disadvantage could be exploited in the preparation of bulk starter cultures, which would be protected for longer periods of time from exposure to pH below 5.0 (Hickey et al., 1983; Mistry and Kosikowski, 1985, 1986). Studies on phage susceptibility in UF retentates have not been reported in the literature.

This study investigated whether UF milk retentates were comparable to ICM and if the same effects could be achieved by increasing the buffering capacity of starter media by increasing the milk solid content. If so, dairy industries manufacturing cheese from UF milk retentates would be able to prepare their bulk starter cultures in

the same medium. This would minimize the adjustment period of the cultures in the cheese vat and would eliminate the need of purchasing special bulk starter culture media. Small dairy industries produce bulk starter cultures once a week, store them at refrigeration temperature and use them for up to 5 days. Results obtained in chapter 2 and 3 showed that 24% RSM and UF milk retentate R3:1 provided similar protection against exposure to low pH as the ICM after 5 days at 2°C. Cultures prepared in ICM were maintained at higher pH levels (pH 5.35 or above) than cultures prepared in 24% RSM and UF retentate R3:1 (pH 4.7 or above) because the maximum buffering capacity for ICM is at 5.5 compared to pH 4.7-5.1 for 24% RSM and R3:1. Results obtained in chapter 4 showed that cultures stored below pH 4.5 in 10% RSM lost much of their activity after 5 days at 2°C. It was observed that this loss of activity was not plasmid mediated but was due to injury of the cells, resulting in an increased lag phase. Reduced proteolytic activity can lead to decreased growth because peptides and amino acids can not be supplied fast enough, thus slowing down cell growth and lactose metabolism. Injury was caused by an accumulation of the lactate ion as opposed to the hydrogen ion. Freezing of fullgrown cultures in bulk culture media for longer storage periods is an attractive alternative to storage at refrigeration temperatures. Neutralization of the cultures prior to freezing at -70°C minimized the loss of proteolytic activity while viability and lactose metabolism were maintained under these conditions.

Not surprisingly, the milk based media had no phage inhibitory properties. Cultures grown in 24% RSM and R3:1 had even higher phage titres than cultures grown in 10% RSM, most likely due to higher cell numbers that were reached in the former media. Phage multiplication was minimal or inhibited in ICM because of high levels of phosphate and citrate buffers which makes calcium unavailable for phage proliferation in this medium. At present, ICM is a more attractive bulk culture medium than either reconstituted skim milk with increased milk solids or UF milk

retentates because of its phage inhibitory properties. However, cultures prepared in ICM are also susceptible to phage attack once inoculated into the cheese vat. Phage multiplication could possibly be inhibited in 24% RSM and R3:1 by the addition of phosphates and citrates to the media to tie up the free calcium. This alternative however, would not have an economic advantage. The cost of ICM is approximately equal to that of 10% RSM because the base of ICM is whey, a biproduct of cheese manufacture. In addition to increased costs due to ultrafiltration of the fluid milk or increasing the milk solid content, the addition of phosphate and citrate buffers is more labour intensive, is subject to human error in measurement and contributes to the overall cost of the bulk starter medium. The effects of added phosphate and citrate to milk medium on the behaviour of starter cultures and the effect it has on the cheesemaking process warrants further investigation.

One of the main advantages of manufacturing cheese from UF retentates is the incorporation of whey proteins into cheese, these proteins are lost during the traditional cheese manufacturing process. This increases the cheese yield and reduces waste water treatment costs by decreasing the biological oxygen demand of the whey. As more dairy industries become involved in manufacturing cheese from UF milk retentates, it would be desirable to use starter cultures prepared from UF milk retentates because their growth substrate would be similar, minimizing the adjustment period in the cheese vat. An additional increase in cheese yield can be obtained because of the addition of proteins present in the retentate bulk culture medium. The use of UF bulk cultures would produce cultures with good activity and high cell numbers.

These studies have shown that starter strains presently used in the Alberta dairy industry are able to grow well in UF retentates and 24% RSM. The substrates provide protection from exposure to low pH and produce active starters with increased cell numbers but need some modification to inhibit phage multiplication. Starter

cultures can be successfully frozen at -70°C in 10% RSM as well as in 24% RSM and R3:1 for at least 2 months without impaired lactose metabolism. Neutralization of the cultures before freezing minimized loss of proteolytic activity.

An interesting observation made when determining the host range of phage strains was the gradual loss of phage susceptibility when cultures were repeatedly subcultured in M17 medium. When $MgSO_4$ was removed from the medium, starter strains remained susceptible to phage. These observations suggest that M17 is not an ideal medium for phage assays.

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